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(54) Title: RAT PLURIPOTENT EMBRYONIC STEM CELLS AND METHOD OF OBTAINING AND USING SAME

(57) Abstract

The present invention provides pluripotent embryonic stem cells derived from rat. These cells are useful in the production of chimeric and transgenic rats. Methods and cell culture media and conditions appropriate for the production of rat pluripotent embryonic stem cells are described, together with details of the morphology enabling recognition of the cells, as well as methods for producing chimeric and transgenic rats.

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RAT PLURIPOTENT EMBRYONIC STEM CELLS AND METHOD OF OBTAINING AND USING SAME

Technical Field of the Invention

The present invention relates to pluripotent embryonic stem cells derived from rat, to a method of obtaining and culturing the rat embryonic stem cells, and to cell culture media and conditions appropriate therefor, as well as to the use of such cells in the production of chimeric and transgenic rats.

Background of the Invention

The traditional genetic approach to the study of gene function as reflected in a particular phenotype was limited to the study of fortuitous mutations, or pre-existing genetic polymorphisms. Under this approach, the only experimental option for increasing the number of genetic variants available for study was mutagenesis followed by time-consuming screening for relevant alleles, or the serendipitous recovery of such alleles. Such an approach, while effective, is quite cumbersome. Moreover, such an approach cannot provide information on lethal mutations, or mutations for which a phenotype is not readily apparent, such as perhaps, certain mutations of protein structure, or of genetic control.

Based on this, an ability to fully realize all genetic possibilities, which is a prerequisite for a true understanding of gene function, as well as manipulation of that function, requires an ability to deliberately modify the gene in a targeted fashion and to directly generate new alleles. This is the theoretical basis of "reverse genetics", which comprises analyzing the function of a gene by deliberately creating mutated versions of the gene, and directly assessing the effect of the newly created alleles on the phenotype of the living organism.

The ability to realize all genetic possibilities for a gene is of particular relevance to research on mammalian

genetic disorders. Animals demonstrating deletions, modifications of function, or overexpression of regulatory or structural genes may be employed in the study of the etiology and clinical manifestations of the disease, as well as in testing potential interventional therapies. A truly rigorous functional analysis requires the establishment of a phenotype for both the gain-of-function and the loss-of-function of the gene. Namely, whereas gain-of-function mutations comprise an increase in the expression or function of a gene, loss-of-function mutations comprise a decrease in expression or function of a gene. In many cases, gain-of-function mutations can be obtained by simple transfection of embryonic stem cells, or microinjection into oocytes of an extra DNA copy of the gene. However, without specific integration of the extra gene into the genome, or its presence in a vector which is capable of autonomous replication, the phenotype produced by the extra copy will not be stable. Similarly, without specific integration of the gene into the stem cells of an organism, the allele will not exhibit Mendelian inheritance. Even more importantly, in gene therapy for defective genes, in some cases it may be necessary to replace the defective gene with the normal gene at the appropriate locus, since for proper expression, the gene may need to be integrated in a region of "active chromatin". Alternatively, such gene replacement may be necessary to maintain the appropriate number of regulatory sequences in the cell by preventing an increased number of gene promoters from diluting out regulatory molecules.

Previously, the technology for targeted gene modification was available for only purportedly 'simple' species such as bacteria or yeast. More recently, the technology has become available for higher-level eukaryotes, e.g., the fruit fly, and has been extended to the mouse. The advent of targeted <u>in vivo</u> gene mutagenesis in mouse was made possible in part by the isolation and establishment in culture of pluripotent cells from <u>in vitro</u>

cultures of mouse blastocysts (Evans et al., Nature, 292, 476-480 (1981)). These pluripotent cells have a normal karyotype and are able to differentiate in vitro, or after inoculation into a mouse. More importantly, these pluripotent cells can be employed as a vehicle for the transfer into the mouse genome of mutant alleles, which are either selected in cell culture, inserted into the cells via transformation with specific DNA fragments, or integrated into the genome of the pluripotent stem cells. Moreover, the ability of these cells to colonize the germ line can be further exploited by coupling this capability with methods for insertional mutagenesis and targeted disruption of specific genes. The resultant phenotype can be examined in the living organism. The use of this approach is exemplified by the creation of null alleles at the murine hypoxanthine phosphoribosyl transferase (HPRT) gene locus (Kuehn et al., Nature, 326, 295 (1987); Hooper et al., Nature, 326, 292 (1987)). Similarly, three separate patents have recently been issued for genetically engineered mice (Journal of Proprietary Rights, "PTO Issues Three Animal Patents", 19 (February 1993)). These patents concern a mouse capable of making human beta interferon, a protein that fights viral infection, a mouse that fails to develop a mature immune system, for use in the study of treatments for AIDS, and a mouse that has an enlarged prostate gland, for study of the treatment of this problem in humans.

Whereas colonization of the embryo including the germ line with pluripotent stem cells can be used to generate a chimeric animal, introduction of exogenous DNA into the pluripotent cells prior to colonization, or insertional mutagenesis of these cells, can be used to generate a transgenic animal. By definition, a transgenic animal is one which possesses an alteration in its DNA as a result of intentional experimental intervention. The production of a transgenic animal may be greatly facilitated if a library of chromosomal genes from the species is available. In

certain species, transgenic animals can be produced by simply microinjecting DNA into the zygote, or by transfecting the embryo with recombinant retroviral vectors incorporating the transgene. However, the use of embryonic stem cells as a vehicle for gene transfer has many advantages over these approaches. In particular, employment of embryonic stem cells allows extensive in vitro genetic manipulation, selection, and screening prior to actual generation of the transgenic animal. Moreover, this approach circumvents the tandem, head-to-tail integration of exogenous DNA at a single chromosomal site which can be observed using other approaches.

While the pluripotent cell route to chimera formation is available for mouse, the approach has been hampered in other species due to an inability to obtain pluripotent cells, or due to an inability to obtain pluripotent cells capable of contributing to chimera formation. Specifically, pluripotent cells have been isolated from mink (Sukoyan et al., Mol. Reprod. Dev., 33, 418-431 (1992)) and hamster (Doetschman et al., Dev. Biol., 127, 224-227 (1988)). The mink cells are apparently limited in their pluripotential capability, as such cells are unable to contribute to chimera formation. The same may also be true for the hamster cells, as so far there have been no published accounts of chimera formation with these stem cells. This raises a question of whether cells of a similar type to those of mouse that are capable of contributing to chimera formation can be isolated from other species. Similarly questionable is whether the methods described for mouse and utilized for hamster will be directly applicable to derivation of stem cells from embryos from other species. The lack of general applicability of the approach to other species is supported by the inability of competent researchers to isolate sheep embryonic cells using the method employed for mouse embryonic cells (Handyside et al., Roux Arch. Dev. Biol., 196, 185-190 (1987)), as well as by the need of researchers

producing pluripotent embryonic stem cells from ungulates, as exemplified by bovine and porcine species, to develop special techniques appropriate for these species (PCT WO 90/03432; McWhir, Isolation and Characterisation of Pluripotential Embryonic Cell Lines in Farm Animals, Ph.D. Thesis (Calgary, Alberta: University of Calgary, 1987)). In other words, subtle and not-so-subtle differences in the rate of growth of pluripotent stem cells, the embryonic stage at which these cells are found, and the tissue culture medium in which the cells can be maintained, as well as in additional properties of the cells, or in the embryologic process in the particular species, can preclude applicability of the approach used in mice to isolate embryonic stem cells to other species, despite even close similarity of the species.

This is unfortunate, as the creation of targeted mutations in the mouse has been an important source of animal models of human disease; however, for many diseases, the physiology of the mouse is either poorly understood or is inappropriate for conducting experiments germane to the particular disease. For example, the study of cardiovascular disease has extensively utilized the rat as opposed to the mouse because of its small size and different physiological responses to experimental manipulations (Thiede, Microsurgical Models in Rat for Transplantation Research, (Berlin: Springer-Verlag, 1985); Burek, Pathology of Aging Rats, (West Palm Beach: CRC Press, 1978)). For similar reasons, the rat is important in the study of lipid transport, atherosclerosis, hypertension, and cardiomyopathy (Storve et al., Biochim. Biophys. Acta, 1167, 175-181 (1993); Sanders et al., Neurosci. Biobehav. Rev., 16, 207-217 (1992)). Detailed information from behavioral studies is much more readily available for the rat than for the mouse (Wenk, ACTA Neurobiol Exp (Warsz), 50, 219-223 (1990); Haracz et al., Neurosci. Biobehav. Rev., 17, 1-12 (1993); Piccoli et al., ACTA Neurol. (Napoli), 14, 455-468 (1992)), and the vast

majority of extant reproductive endocrinological data has been established in the rat (Altman et al., In: Inbred and Genetically Defined Strains of Laboratory Animals. Part 1: Mouse and Rats, (Bethesda: FASEB, 1992) 340-348). Furthermore, the response to carcinogens is well characterized in the rat, and this species is critical in many cancer models (Reddy et al., In: Mechanisms of Carcinogenesis in Risk Identification, H. Vainio, P.N. Magee, D.B. McGregor, and A.J. McMichael, eds. (Lyon, France: International Agency for Research on Cancer, 1992) 225-235). Based on this, the ability to establish a pluripotent stem cell population from the rat, and to make chimeras with the pluripotent cells, would prove a great asset to the study of mammalian diseases and disease states for which the rat presents a more appropriate model than murine or ungulate species.

There remains a need, therefore, for pluripotent embryonic stem cells from the rat. It is an object of the present invention to provide such stem cells, as well as to provide a method of obtaining and culturing the cells, and a method of using such cells in the production of chimeric and, in particular, transgenic rats. These and other objects and advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

Brief Summary of the Invention

The present invention provides pluripotent embryonic stem cells derived from rat. These cells are capable of prolonged growth in culture in the absence of overt differentiation. Methods and cell culture media and conditions appropriate for the isolation of the cells, as well as morphological details enabling recognition of the cells, are provided herein.

The present invention also provides methods and cell culture media and conditions for the maintenance of the pluripotent embryonic stem cells in vitro. Under

appropriate culture conditions, however, the cells are capable of differentiation into an array of cell types which predominate in the developing embryo. This propensity of the embryonic stem cells attests to their pluripotent nature. Accordingly, the present invention further provides methods for inducing differentiation of the pluripotent embryonic stem cells.

The pluripotent nature of the embryonic stem cells is further corroborated by their ability to contribute to chimera formation. The present invention, therefore, further provides methods for chimera production, as well as methods for the generation of transgenic rats.

Brief Description of the Drawings

Figures 1A-H are a series of photomicrographs demonstrating the various stages of rat embryonic stem cell (RESC-01) isolation: (A) attached rat blastocysts, T = trophoectoderm, I = inner cell mass, F = HREF feeder layer (phase contrast, bar = 100 μm); (B) flat embryonic stem cell colony (ES) surrounded by endoderm cells (En) (Nomarski optics, bar = 40 μm); (C) colonies of rat embryonic stem cells following mechanical disruption (phase contrast, bar from A = 200 μm); (E) endoderm differentiation from RESC-01 cell colonies (phase contrast, bar from A = 200 μm); (D & F) higher magnification showing endoderm differentiation (Nomarski optics, bar = 20 μm); (G) simple embryoid bodies (phase contrast, bar = 250 μm); (H) cystic embryoid bodies, En = endoderm, Ec = ectoderm (phase contrast, bar = 250 μm).

Figure 2 is a graph of time (day) versus cell number (x 10^5) for RESC-01 cells grown on HREF embryonic fibroblasts (solid line), STO mouse embryonic fibroblasts (long dashes), gelatin-coated plastic (stippled line), or plastic (short dashes). The growth curves were obtained by plating RESC-01 cells in the presence of 500 units/ml of LIF.

Figure 3 is a graph of time (day) versus differentiated colonies (%) for RESC-01 cells grown on HREF embryonic fibroblasts (solid line), STO mouse embryonic fibroblasts (long dashes), gelatin-coated plastic (stippled line), or plastic (short dashes).

Figures 4A-B are graphs of time (day) versus cell number (x 10^5) for RESC-01 cells grown on HREF embryonic fibroblasts (A) and gelatin-coated plastic (B). The growth curves were obtained by plating RESC-01 cells in the presence of a LIF concentration of 0, 100, 500, 1000 or 2000 units/ml.

Figures 5A-B are graphs of time (day) versus differentiated colonies (%) for RESC-01 cells grown on HREF embryonic fibroblasts (A) and gelatin-coated plastic (B). RESC-01 cells were plated in the presence of a LIF concentration of 0, 100, 500, 1000 or 2000 units/ml.

Figures 6A-C are a series of photomicrographs demonstrating expansion of the rat blastocyst: (A) rat blastocyst at 4.5 days post coitus (sperm discovery = day 0.5; blastocyst diameter = 100 μm); (B) blastocyst of A rotated 90°; (C) fully expanded rat blastocyst isolated about 4 hours after the appropriate treatment of the blastocyst in A (blastocyst diameter = 100 μm). An injection pipette shown on the right contains a RESC-01 cell.

Figures 7A-D are a series of photographs demonstrating chimeras produced by the injection of RESC-01 cells into Holtzman strain rat blastocysts: (A) Chimera 5001 (age = 40 days); (B) Chimera 5000 (age = 40 days); (C) Chimera 5002 (age = 35 days); (D) Chimera 4999 (age = 9 days) as well as a non-chimeric sibling (top portion of the photograph) exhibiting the characteristic Holtzman albino coat color.

Detailed Description of the Invention

In general, mammalian embryogenesis proceeds in a remarkably similar fashion across species. However, by the time the embryos of different species are at a

developmental stage at which implantation can ensue, numerous species-specific differences in development can be tabulated. While a single variation between species may appear subtle and of little consequence, when considered in the aggregate, such variations evince dramatic species-specific differences in embryogenesis. Accordingly, while procedures for the isolation of embryonic stem cells and use of such cells for the production of chimeric or transgenic animals may have been developed for other species, important rat-specific differences in embryologic development preclude the verbatim application of such procedures for the isolation of rat pluripotent embryonic stem cells and for the production of chimeric and transgenic rats.

In particular, several factors have been identified which, while seemingly of little import when considered individually, when considered in the aggregate, appear to contribute substantially to the ability to obtain and culture pluripotent rat embryonic stem cells, and to use such cells in the production of chimeric, and potentially transgenic, rats.

For example, timing is a critical concern in the present invention, in terms of the appropriate time for isolation of embryos from which pluripotent embryonic stem cells can be obtained, the length of time such embryos should be maintained in culture prior to isolation of cells, and even down to the smallest detail of recognizing when to passage or refeed cells. Similarly, tissue culture conditions are of importance in this invention, not only for enhancing the efficiency of pluripotent rat embryonic stem cell isolation, and incorporation of such cells into an early stage embryo, but also for maintaining embryonic stem cells in an undifferentiated state. Moreoever, recognition of the different development stages and appropriate manipulation at each stage is a relevant factor in the present invention. In particular, one of the key elements in embryonic stem cell isolation is to disrupt

development of the isolated embryo prior to extensive differentiation, but at a point when the stem cell component is sufficiently large to survive.

Furthermore, while these factors apparently contribute to the present invention, other factors not particularly highlighted, and yet encompassed by the methods, descriptions and compositions disclosed herein, most likely similarly contribute to the ability to obtain and culture pluripotent rat embryonic stem cells, and to employ these cells in chimeric and transgenic rat generation.

Accordingly, the present invention provides, among other things, substantially pure pluripotent embryonic stem cells from rat. More specifically, the present inventive embryonic stem cells are obtained from a preimplantation embryo. In the context of the present invention, a "preimplantation embryo" is an organism in an early stage of development occurring in the period immediately following fertilization of the egg, up until implantation into the wall of the uterus, such as, for example, the eight-cell, morula or blastocyst stage. Similarly, an "embryonic cell" is any cell that can be obtained from such a preimplantation embryo.

The present invention also provides a method for obtaining the rat pluripotent embryonic stem cells from a preimplantation embryo. Since the appropriate timing for isolation of the preimplantation embryo, as well as isolation of putative stem cells from this embryo, is important to this invention, the present invention accordingly provides information concerning the stage of development at which the preimplantation embryo can be isolated and placed in culture for the purpose of isolating pluripotent embryonic stem cells, as well as the length of time of maintaining the embryo culture which is sufficient to allow the cultured preimplantation embryo to obtain an appropriate size and stage of development from which potential stem cells can be separated by disruption, and the manner in which this disruption and subsequent culture

of disrupted fragments is to be conducted. In a preferred method of the present invention, the preimplantation embryo is a blastocyst.

Specifically, the substantially pure pluripotent embryonic stem cells are obtained by removing a preimplantation embryo, preferably a blastocyst, from a rat uterus. A rat blastocyst obtained between days 4 and 5 of pregnancy, particularly day 4.5, is at an appropriate developmental stage to allow isolation of pluripotent embryonic stem cells. This is in distinct contrast to other species, in which embryonic development proceeds differently than in rat, resulting in a difference in time when preimplantation embryos are isolated. For example, in mouse and hamster, embryos appropriate for isolation of stem cells are obtained on day 3.5 of pregnancy (Doetschman et al., Dev. Biol., 127, 224-227 (1988); Robertson, In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E.J. Robertson, ed. (Oxford: IRL Press, 1987) 71-112), and in bovine and porcine species, embryos appropriate for isolation of stem cells are obtained after the sixth day of pregnancy (PCT WO 90/03432).

The culture requirements of the isolated preimplantation embryos are also important in the context of the present invention. Accordingly, the present invention provides preferred culture conditions, for example, a preferred feeder cell layer, which are appropriate to employ for obtaining rat pluripotent embryonic stem cells from isolated preimplantation embryos. The invention also provides conditions for maintaining such cells in culture in the absence of cell differentiation. A preferred method of culturing embryonic stem cells comprises maintaining the cells in the presence of leukemia inhibitory factor (LIF), or culturing the cells on a feeder cell layer. LIF has been shown to inhibit the differentiation of mouse embryonic stem cells in culture, even in the absence of embryonic fibroblast feeder layers (Pease et al., Exp. Cell Res., 190, 209-211 (1990); Mummery

et al., Cell Differ. Dev., 30, 195-206 (1990); Brown et al., In Vitro Cell. Dev. Biol., 28A, 773-778 (1992)). While a preferred feeder cell layer of the present invention is comprised of rat embryonic fibroblasts, one skilled in the art will recognize that additional means and agents can similarly be utilized to impede differentiation of rat pluripotent embryonic stem cells.

The isolation of rat stem cells is facilitated through use of a feeder cell layer for culturing rat blastocysts which differs from the feeder cell layers employed for murine, hamster, and ungulate species. Specifically, the feeder layers on which the blastocysts are placed is preferably comprised of primary embryonic fibroblasts isolated from midgestation Holtzman strain fetuses (i.e., obtained on the 14th day of pregnancy) by maceration and trypsin treatment of the embryo carcass. The HREF (Holtzman strain Rat Embryonic Fibroblast) cells are maintained under suitable conditions, e.g., in a 5% CO_2 atmosphere at 37°C in DMEM containing 10% FBS (Intergen Co.), 2 mM L-glutamine and penicillin/streptomycin. Growth arrest of the fibroblasts may be achieved by any suitable means, e.g., incubating the cells with fresh medium containing mitomycin-C (10 µg/ml; Sigma, St. Louis, MO, M-0503) for 4 hours. The cells may then be plated after suitable exposure to the growth arrest medium, e.g., 24 hours after exposure to mitomycin-C, at a suitable density.

Thus, the preimplantation embryos, particularly blastocysts, which are removed from the rats are placed on rat embryonic fibroblast feeder layers in appropriate culture dishes, e.g., organ culture dishes. A medium needs to be employed for in vitro culture which will facilitate the growth of the preimplantation embryos, e.g., blastocysts (Van Winkle et al., Dev. Biol., 142, 184-193 (1990); Ng et al., In: Current Topics in Developmental Biology, R.A. Pederson, ed. (San Diego: Academic Press, 1992) 235-274). A cell culture medium such as Markert's modification of Whittingham's medium (Yamamura et al., Dev.

Genet., 2, 131-146 (1981)) is preferred, particularly when further supplemented with 20% FBS (lot-screened for mouse ES cell growth, Intergen Co., Purchase, NY), penicillin (100 units/ml)/streptomycin (100 mg/ml), 1% non-essential amino acids (NEAA, 100X stock from GIBCO BRL, Gaithersburg, MD, #320-1140PG), 1% nucleosides from stock (100X stock = adenosine 3.0 M, guanosine 3.0 M, cytidine 2.3 M, uridine 3.0 M, thymidine 1.0 M), 1% β-mercaptoethanol stock (stock = 7 μl/10 ml in phosphate buffered saline), and LIF (2000 units/ml ESGRO™, GIBCO BRL). Alternatively, a cell culture medium such as DMEM supplemented as indicated for Markert's modification of Whittingham's medium may be employed to replace the supplemented version of Markert's modification of Whittingham's medium, either entirely, or only in the later stages of isolation of rat stem cells. These media, which were found to be appropriate for growth of rat blastocysts and isolation of rat stem cells, are different from the supplemented DMEM medium that is employed for isolation of murine (Robertson, In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E.J. Robertson, ed. (Oxford: IRL Press, 1987) 71-112) and ungulate stem cells (PCT WO 90/03432).

The preimplantation embryos, e.g., blastocysts, attach within a reasonable period of culturing, e.g., within about 48 hours of culturing the blastocysts, and then hatch from the zona pellucida. The medium is preferably changed every day after the first 48 hours of culturing. DMEM supplemented as indicated previously for Markert's modification of Whittingham's medium has been found to be best suited for sustaining rat embryo growth at this stage, as evidenced by the increased survival and decreased differentiation of rat blastocysts cultured using DMEM.

After a suitable period of time, generally about 72 hours, some of the attached blastocysts will have expanded inner cell mass (ICM) populations. Between that time and another about 12-24 hours, i.e., between about 72 and 94 hours of incubation, the rat blastocysts will have

typically achieved an appropriate size and level of development to allow disaggregation of an ICM-derived component. It is important to isolate ICM cells only after the rat blastocysts have reached a stage where substantial ICM proliferation has occurred but cells have not yet differentiated into endoderm cells. Thus, the ICM outgrowth will preferably be disrupted by suitable means, e.g., by pipetting, at about 96 hours following incubation. The cells are preferably fed a few hours, e.g., about two hours, prior to this disruption. Alternatively, the ICM outgrowth can be dissociated with use of trypsin, as is done for murine and ungulate species. However, this is not a preferred means of dissociation, as trypsinization of rat blastocysts, for a reason which is unclear, deleteriously impacts upon the ability to isolate pluripotent stem cells from the rat blastocyst fragments. Accordingly, in the case of the rat, trypsinization needs to be done with extreme caution.

The individual disrupted ICM outgrowths are then transferred to separate dishes, e.g., organ culture dishes, precoated with HREF feeder layers and are maintained in supplemented DMEM. Additional fresh medium is added as needed, e.g., about every 24 hours, and the medium is preferably changed every 48 hours. The growing colonies derived from the initial ICM are disrupted, preferably mechanically, over a suitable period of time, e.g., every day within the same dish for 5 days. This repetitive disruption, preferably mechanical disruption, of colonies derived from the ICM is yet another step which appears important in the isolation of rat embryonic stem cells, but not stem cells from other species.

When a significant portion, e.g., about 50%, of the HREF feeder layers are covered with colonies, single cell suspensions of the embryonic cells from the rat blastocysts are made with trypsin and placed on new HREF feeder layers. The rat embryonic cells are expanded to a suitable level,

e.g., about 75% confluence, trypsinized, and passaged on HREF feeder layers in suitable tissue culture plates.

The cells can be frozen in freezing vials in a suitable medium, e.g., Gibco freezing medium, after the addition of fresh DMEM medium, preferably about 2 hours after the addition of fresh DMEM medium. The cultures are preferably refed a suitable period of time, e.g., 2 hours, prior to exposure to trypsin. The cells may also be frozen on plates by feeding cells with supplemented DMEM and then, after a suitable period of time, e.g., 2 hours later, replacing the DMEM with a suitable freezing medium, e.g., with about 400 μl of Gibco freezing medium. The plates should be tightly wrapped in thin plastic film and stored at a suitably low temperature, e.g., in a -70°C freezer. The cells may be thawed by any suitable means, e.g., by adding 600 μl of prewarmed DMEM medium, immediately aspirating off the medium, and adding 1 ml of prewarmed DMEM medium.

The resultant rat embryonic stem cells derived from rat blastocysts are typically rather small, e.g., about 10-20 microns across, and are flat-appearing when observed with Nomarski optics. Under optimal culture conditions differentiation of the embryonic stem cells does not occur. The rat embryonic stem cells have a prominent nucleus containing one or more nucleoli and typically contain a minimal amount of cytoplasm. The cells can be demonstrated to be diploid by karyotype analysis, and there are no obvious borders between the cells in culture.

The identification of the rat embryonic stem cells may be validated by carefully observing the growth of the putative colony. In particular, the preferred rat embryonic stem cell colony of the present invention exhibits growth and an absence of overt differentiation, as well as an ability to contribute to chimera formation. These characteristics of the rat embryonic stem cells are indicative of pluripotent stem cells.

The rat pluripotent embryonic stem cells of the present invention may be maintained in culture and grown on various substrata. The stem cells can be plated onto feeder layers in DMEM supplemented as previously described for the isolation of rat embryonic stem cells. The stem cells are preferably grown on HREF fibroblast feeder layers, since, while they grow on STO mouse fibroblast feeder layers, as well as on gelatin-coated plastic and even plastic, they do not grow as well on these other materials. The present inventive rat embryonic stem cells can be maintained in culture in the undifferentiated state using highly purified LIF. The proportion of differentiated rat embryonic stem cells decreases as the concentration of LIF is increased, particularly when the stem cells are plated on HREF feeder layers, as LIF more effectively retards differentiation when the rat embryonic stem cells are plated on HREF feeder layers, without, however, influencing the proliferation of the HREF embryonic fibroblasts. In the absence of LIF, when cultured on the feeder layers, the stem cells demonstrate slow differentiation toward endoderm-like cells. This propensity of the stem cells for endoderm differentiation despite the influence of the fibroblast feeder layer appears not to have been reported for the embryonic stem cells from other species. Accordingly, the supplemented DMEM used in the present invention preferably contains a suitable amount of LIF, e.g., at least about 500 units/ml LIF, preferably at least about 1000 units/ml LIF, most preferably at least about 2000 units/ml LIF, and as high as 10000 units/ml LIF or more. The apparent necessity of such high concentrations of LIF to retard differentiation has also not been reported for other species.

The present inventive rat pluripotent embryonic cells are useful in that they can be employed to generate chimeric, as well as transgenic, rats. One skilled in the art will recognize that selection of the rat strain to use for chimera formation is important, but that a variety of

strains may be suitably employed, both in terms of the strain appropriate for embryonic stem cell isolation and the strain appropriate for isolation of the preimplantation embryo into which the embryonic stem cells will be incorporated. Thus, the present invention provides a preimplantation embryo or embryonic cell into which one or more of the rat embryonic stem cells, or nucleuses of the cells, have been introduced, as well as a method of incorporating one or more rat pluripotent embryonic stem cells into a rat preimplantation embryo and, in particular, a rat blastocyst. The present invention also provides a chimeric or transgenic rat which is the progeny of such a preimplantation embryo or embryonic cell.

The introduction of the present inventive rat embryonic stem cells, or nucleuses of the cells, into a preimplantation embryo or embryonic cell may be accomplished by any suitable means. For example, the stem cells may be injected into rat blastocoel cavities as described for injection into mouse blastocoel cavities (Bradley, In: Teratocarcinomas and embryonic stem cells: a practical approach, E.J. Robertson, ed. (Oxford: IRL Press, 1987) 113-151), with necessary modifications being made to accommodate species-specific differences in embryological processes. In particular, rat blastocysts appropriate for injection are obtained on day 4.5 of pregnancy as compared with day 3.5 for mice. In addition, the rat morula stage embryo differs distinctly from that of the mouse in being planar in shape (Yamamura et al., Dev. Genet., 2, 131-146 (1981); Weinberg et al., J. Cell Sci., 89, 423-431 (1988)). This geometry is maintained in the rat blastocyst which is characteristically ovoid in shape. Whereas the mouse blastocyst undergoes a period of prolonged expansion prior to implantation in the uterus, the rat blastocyst implants quite rapidly following a short period of expansion. Accordingly, based on the characteristic ovoid shape of blastocysts isolated from the rat, it may be necessary to expand the blastocyst prior to

microinjection of the stem cells, e.g., for rat chimera formation.

The expansion of rat preimplantation embryos, especially blastocysts, prior to introduction of the embryonic stem cells may be accomplished by any suitable means, preferably by incubation in a cell culture medium comprised of a suitable carbon source, minerals, buffers, proteins, carboxylic acids, and carboxylic acid derivatives. The expansion is most preferably effected by incubating the blastocyst at 37°C in 5% CO_2 atmosphere for about two hours in Brinster's medium (Brinster, In: Pathways to Conception (Springfield: Charles G. Thomas Publishing Company, 1971)) that has been modified to facilitate expansion of the rat blastocyst, preferably by adding with stirring to 75 ml of nanopure water in a 100 ml volumetric flask the following tissue culture grade reagents: 554.5 mg NaCl, 35.6 mg KCl, 16.2 mg KH_2PO_4, 14.3 mg anhydrous $MgSO_4$, 210.5 mg $NaHCO_3$, 100 mg dextrose, 5 mg streptomycin sulfate, 6 mg penicillin G, 100 mg albumin (BSA Pentax fraction V), 5.6 mg sodium pyruvate, and 0.38 ml of D,L-sodium lactate. Following the addition of 18.8 mg of $CaCl_2$, the medium is brought to a final volume of 100 ml. For microinjection, the expanded blastocysts are placed in the microdrop of the medium along with the stem cells. A suitable number of stem cells, e.g., about 10 to 30 stem cells, can be injected into the blastocoel using conventional means, preferably a DeFunbrune pump.

While this method of expansion and injection is most preferred in the context of the present invention based on certain peculiarities in the rat embryologic process, one skilled in the art will recognize that there exist alternative means of expanding the rat blastocyst or preimplantation embryo, as well as alternative means of incorporating the cells into a preimplantation embryo, particularly a blastocyst.

For example, in another preferred method of the present invention, one or more pluripotent embryonic stem

cells may be incorporated into a rat embryo by coculturing a rat preimplantation embryo with the pluripotent embryonic stem cells. This preferred method is similar in certain respects to an approach used in mice (Wood et al., Proc. Natl. Acad. Sci., 90, 4582-85 (1993)). The cells are preferably prepared in supplemented DMEM medium containing LIF, for example, in a concentration of at least 500 units/ml, most preferably in a concentration of about 2000 units/ml, to which is then added the rat embryos, preferably morula stage rat embryos isolated from pregnant rats at day 3.5 of pregnancy. After a suitable number of embryonic stem cells are observed attached to the embryos, the embryos are preferably cultured in a suitable medium, e.g., in particular in an organ culture dish coated with heat-inactivated rat serum and containing Markert's modification of Whittingham's medium that had been adjusted with water to an osmolality of 295 mOsm/kg H_2O. After a suitable period of culturing time, e.g., about 20-30 hours, rat embryos are obtained to which have been introduced the embryonic stem cells of the present invention.

In yet another preferred method of the present invention, a nucleus of a pluripotent embryonic cell may be introduced into a rat embryonic cell by replacing the pronuclei of a fertilized rat egg with the nucleus from the pluripotent embryonic stem cell. Following development of the egg in culture, the preimplantation embryo could be transferred to a pseudopregnant surrogate mother. This approach has been suggested for mouse (Palmiter et al., Ann. Rev. Genet., 20, 465-99 (1986)).

Alternative approaches such as these, as well as additional approaches not specifically recited, may be employed to introduce rat embryonic stem cells into the rat preimplantation embryo in the event that strain-specific differences, or other factors, render the method of expansion of the preimplantion embryo and, in particular, the method of expansion of the blastocyst, ineffective for a particular strain.

After the incorporation of the embryonic stem cells into preimplantation embryos, particularly blastocysts, the preimplantation embryos are transferred to the uteri of surrogate mothers on day 3 or 4, preferably day 3.5, of pseudopregnancy of the rats. This is another example of a difference between the rat and other species, inasmuch as such transfer is routinely performed on day 2.5 for the mouse.

Since, prior to the present invention, less was known in the rat as compared with other species about appropriate timing for embryo transfer, techniques for both inducing and dating pseudopregnancy in the rat needed to be developed. Pseudopregnancy in the surrogate rats may be established by a variety of means, preferably by the mechanical stimulation of the cervix during estrus (Ng et al., In: Current Topics in Developmental Biology, R.A. Pederson, ed. (San Diego: Academic Press, 1992) 235-274). Alternatively, pseudopregnancy can be established as for mice, by mating female rats with sterile males and selecting females exhibiting vaginal copulation plugs (plug discovery = day 0.5). The timing of pseudopregnancy can be established by noting the day of onset of leukocytes in the vaginal smear (Ng et al., supra). These novel methodologies developed expressly for the rat facilitated rat chimera production by providing greater control over the manipulation of embryologic stages. It has been established that methods to reliably establish and date pseudopregnancy in the rat will impact upon the ability to produce chimeras with high efficiency (Weinberg et al., Science, 227, 524-527 (1985); Iannaccone et al., Development, 99, 187-196 (1987); Iannaccone et al., J. Exp. Zool., 243, 217-223 (1987); Iannaccone et al., In: Banbury Report 26: Developmental Toxicology: Mechanism and Risk, J.M. McLachlan, R.M. Pratt, and C.L. Markert, eds. (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory, 1987) 73-92; Iannaccone et al., Int. J. Cancer, 39, 778-784 (1987); Iannaccone et al., Cell Differ. Dev., 25, 77-90

(1988); Weinberg et al., J. Cell Sci., 89, 423-431 (1988); Iannaccone, FASEB J., 4, 1508-1512 (1990); Ng et al., In: Current Topics in Developmental Biology, R.A. Pederson, ed. (San Diego: Academic Press, 1992) 235-274; Ng et al., Dev. Biol., 151, 419-430 (1992); Weinberg et al., In: The Role of Cell Types in Hepatocarcinogenesis, A.E. Sirica, ed. (Boca Raton: CRC Press, 1992) 29-53).

After transferring to the uterus of a pseudopregnant rat a preimplantation embryo or an embryonic cell into which has been introduced one or more pluripotent embryonic stem cells or nucleuses of the cells, the surrogate mothers may be allowed to deliver naturally, thereby resulting in the preparation of rat chimeras. When the embryonic stem cells have colonized the germ line, germ line chimeras are obtained as a result of the present invention; otherwise, somatic cell chimeras are obtained.

The present invention also provides for the modification of the rat pluripotent embryonic stem cells prior to introduction into the preimplantation embryo, especially for the purpose of preparing transgenic rats which are the progeny of such embryos. In particular, the rat embryonic stem cells can be modified prior to incorporation, and appropriate screens can be conducted to select for rat embryonic stem cells exhibiting the desired properties. For example, a genetic marker can be introduced into the rat embryonic stem cells, or the cells can be infected with viruses, or treated with viral, chemical, or physical agents which alter certain properties of the cells. Alternatively, the rat embryonic stem cells can be fused with cells from another species.

Accordingly, the present invention provides a preimplantation embryo into which has been introduced rat pluripotent embryonic stem cells which comprise genetic material with at least one change therein, as well as an embryonic cell into which has been introduced a nucleus of such a rat pluripotent embryonic stem cell which comprises genetic material with at least one change therein. One

skilled in the art will recognize that the genetic material of rats is comprised of deoxyribonucleic acid (DNA). Ribonucleic acid (RNA), as well as oligonucleotides, to the extent that they impact on gene expression, can also be considered genetic material. Accordingly, the present invention also contemplates so-called antisense and triple helix DNA approaches, as well as other approaches, including use of ribozymes, particularly those which act in a sequence-specific fashion, which exert an effect, albeit a transient one, on gene expression. In the context of the present invention, gene expression is defined as including any stage or activity from transcription of nascent mRNA to appropriate modification and transport of translated protein, such as, for example, elongation of initiated message or translocation of nascent message from the nucleus to the cytoplasm.

In a preferred method of the present invention, the change in genetic material is selected from the group consisting of addition of a DNA segment, rearrangement of a DNA segment, deletion of a DNA segment, replacement of a DNA segment, methylation of unmethylated DNA, demethylation of methylated DNA, and introduction of a DNA lesion.

The DNA segment can be as small as one nucleotide, can be single-stranded or double-stranded, and can be a mixture of single-stranded and double-stranded regions. Similarly, the addition of a DNA segment to the rat pluripotential embryonic stem cell can be done by the actual physical integration of the segment into the genome as well as by introduction of the segment in an autonomously replicating vector, as is known in the art. Such addition can be accomplished using molecular or genetic techniques, or a combination of techniques. Similarly, a DNA lesion can include but is not limited to a missing base or altered base (e.g., an alkylated base), a cyclobutyl dimer, DNA strand breaks, and cross-linking of DNA strands.

Moreover, the artisan will be familiar with state-of-the art approaches for generating a change in genetic

material which can be applied to the rat pluripotent embryonic stem cell of the present invention. For example, in terms of molecular approaches, rat embryonic stem cells can be transfected with mammalian expression vectors, enhancer trap vectors, promoter-probe vectors, vectors in which the subcloned DNA is under the control of its own cis-acting regulatory elements, and vectors which are designed to facilitate gene integration or gene replacement in host cells.

One predominant means of targeted gene integration or replacement is through homologous recombination using a vector which replaces normal gene sequences with an engineered gene that may, for example, contain a premature transcriptional stop signal, or takes the coding sequence of the gene out of frame. Since homologous recombination requires correspondence of portions of the exogenous DNA with segments of the endogenous DNA (i.e., correspondence of segments flanking both 5′ and 3′ ends of the gene for double-strand crossover events resulting in gene replacement, and correspondence with segments flanking either the 5′ or 3′ end of the gene for single-strand crossover events resulting in gene integration), homologous recombination is facilitated using a gene or chromosomal library of genes subcloned into a vector containing portions of the long and short arm of the chromosome which flank the relevant gene, as well as containing an additional selectable gene which confers upon host cells some particular characteristic, such as, for example, antibiotic resistance. The present invention also contemplates untargeted mutagenesis, as, for example, by appropriate treatment with mutagens. Additionally, any technique for mutagenesis known in the art can be used, including but not limited to, in vitro site-directed mutagenesis (Hutchinson et al., J. Biol. Chem., 253, 6551 (1978)), as well as any commercial kit or product for mutagenesis.

Similarly, a DNA sequence may or may not be subcloned into a vector used for transfection. Potential DNA/ sequences which may be present include but are not limited to: coding sequences for structural or regulatory genes and non-coding sequences important in the regulation of gene expression, or important in the processing or transport of nascent DNA or protein. The DNA sequences may be those found in nature or may be entirely or partly engineered. Alternatively, the introduced nucleic acid may be RNA.

Any means, including any type of plasmid or non-plasmid vector, such as a cosmid or modified virus, may be employed to introduce the DNA sequence into the rat pluripotent embryonic stem cells. Alternatively, the DNA may be introduced as a liposome-DNA complex or attached to an adenoviral capsid. The vector system must be compatible with the rat pluripotent embryonic stem cells. Vectors can be introduced into the stem cells via transformation, transfection, infection, electroporation, etc.

Accordingly, the present invention provides a method of making a preimplantation embryo into which has been introduced rat pluripotent embryonic stem cells which comprise genetic material with at least one change therein. Similarly, the present invention provides a method of making a rat embryo into which has been introduced rat pluripotent embryonic stem cells which comprise genetic material with at least one change therein. Also, the present invention provides a method of introducing into a rat embryonic cell a nucleus of a rat pluripotent embryonic stem cell which comprises genetic material with at least one change therein.

Moreover, the present invention includes the method of producing a chimeric or, more specifically, transgenic rat by transferring the preimplantation embryo, particularly the blastocyst, or transferring the embryonic cell, to which the rat embryonic stem cells, or nuclei of these cells containing altered genetic material have been

incorporated, to the uteri of pseudopregnant rats. The techniques appropriate for both inducing and dating pseudopregnancy in the rat, as well as the timing of embryo transfer, for such altered embryonic stem cells are the same as for the unaltered embryonic stem cells.

Additionally, the present invention provides a method of producing a rat containing a particular allele in the homozygous state, e.g., rats which are homozygous for DNA sequences introduced into or altered in the rat pluripotent embryonic stem cells. Such rats can be produced by mating with each other chimeric rats which have been produced using the aforesaid methods of the present invention and which are germ line chimeras. The skilled artisan will know the appropriate breeding experiments to perform to verify germ line chimerism. In the event that the allele is lethal when homozygous, as for example are certain mutations of essential genes, the allele can be maintained in the heterozygous state.

The following examples further illustrate the present invention and, of course, should not be construed as in any way limiting its scope.

Example 1

This example confirms that the blastocyst of the rat can be manipulated to give rise to a culture of pluripotent stem cells which can be maintained in vitro.

Pluripotent stem cells were derived from blastocysts obtained from the inbred PVG strain of black-hooded rats (Festing et al., Transplantation, 16, 221-245 (1973)) carrying the *RT1*^{c} haplotype. PVG rats were used because of the availability of congenic strains which can be distinguished at a major histocompatibility class I locus by using particular monoclonal antibodies (Howard et al., Immunology, 41, 131-141 (1980)). Moreover, this strain can easily be distinguished visually from the strain selected as host for chimera formation.

The blastocysts (PVG-*RT1*^{c} x PVG-*RT1*^{c}) were removed from the rat uterus on day 4.5 of pregnancy by sacrificing the animal and flushing out the uterine horns with a balanced salt solution (T6', which may alternatively be designated T6 or T6'M310; Van Winkle et al., Dev. Biol., 142, 184-193 (1990); Ng et al., In: Current Topics in Developmental Biology, R.A. Pederson, ed. (San Diego: Academic Press, 1992) 235-274).

The blastocysts were placed on rat embryonic fibroblast feeder layers in organ culture dishes using rat embryo medium, particularly Markert's modification of Whittingham's medium (Yamamura et al., Dev. Genet., 2, 131-146 (1981)) supplemented with 20% FBS (lot-screened for mouse ES cell growth, Intergen Co., Purchase, NY), penicillin (100 units/ml)/streptomycin (100 mg/ml), 1% non-essential amino acids (NEAA, 100X stock from GIBCO BRL, Gaithersburg, MD, #320-1140PG), 1% nucleosides from stock (100X stock = adenosine 3.0 M, guanosine 3.0 M, cytidine 2.3 M, uridine 3.0 M, thymidine 1.0 M), 1% β-mercaptoethanol stock (stock = 7 μl/10 ml in phosphate buffered saline), and leukemia inhibitory factor (LIF; 2000 units/ml ESGRO^{TM}, GIBCO BRL).

The feeder layers on which the blastocysts were placed were comprised of primary embryonic fibroblasts isolated from midgestation Holtzman strain fetuses (i.e., obtained on the 14th day of pregnancy) by maceration and trypsin treatment of the embryo carcass. The HREF cells were maintained in a 5% CO_2 atmosphere at 37°C in DMEM containing 10% FBS (Intergen Co.), 2 mM L-glutamine and penicillin/streptomycin. Growth arrest of the fibroblasts was achieved by incubating the cells with fresh medium containing mitomycin-C (10 μg/ml; Sigma, St. Louis, MO, M-0503) for 4 hours. The cells were then plated 24 hours after exposure to mitomycin-C at a density of about 5 x 10^5 cells per 60 mm culture dish. The feeder layers were at a density of about 10^6 cells per culture dish at the time of coculturing with blastocysts.

The blastocysts attached within 48 hours of culturing, and subsequently hatched from the zona pellucida. The medium was changed every day after the first 48 hours of culturing, with the medium being DMEM supplemented as indicated previously for Markert's modification of Whittingham's medium. This medium was employed in all subsequent examples, with only the concentration of LIF being varied as indicated. Moreover, it is conceivable that this medium may be employed even in the initial stages of rat embryonic stem cell isolation, in replacement of Markert's modification of Whittingham's medium.

After 72 hours, some of the attached blastocysts had expanded inner cell mass (ICM) populations as exhibited in **Figure 1A**. Between 72 and 94 hours of incubation, the rat blastocysts had achieved an appropriate size and level of development to allow disaggregation of an ICM-derived component, e.g., the rat blastocysts had reached a stage where substantial ICM proliferation had occurred but the cells had not yet differentiated into endoderm cells. The ICM outgrowth was disrupted by pipetting at about 96 hours following incubation. The cells were fed two hours prior to this disruption.

The individual disrupted ICM outgrowths were transferred to separate organ culture dishes precoated with HREF feeder layers, and were maintained in supplemented DMEM. Additional fresh medium was added every 24 hours, and the medium was changed every 48 hours. The growing colonies derived from the initial ICM were mechanically disrupted every day within the same dish for 5 days. The appearance of the colonies following this mechanical disruption is shown in **Figure 1C**.

When 50% of the HREF feeder layers were covered with colonies, single cell suspensions of the embryonic cells from the rat blastocysts were made with trypsin and placed on new HREF feeder layers. The rat embryonic cells were expanded to 75% confluence, trypsinized, and passaged on

HREF feeder layers in tissue culture plates of up to 60 mm diameter.

Cells were frozen in freezing vials in Gibco freezing medium 2 hours after the addition of fresh DMEM medium. The cultures were routinely refed 2 hours prior to exposure to trypsin. Cells were also frozen on plates by feeding cells with supplemented DMEM and 2 hours later, replacing the DMEM with 400 µl of Gibco freezing medium. The plates were tightly wrapped in thin plastic film and stored in a -70°C freezer. Cells were thawed by adding 600 µl of prewarmed DMEM medium, immediately aspirating off the medium, and adding 1 ml of prewarmed DMEM medium.

The resultant rat embryonic stem cell line derived from the blastocyst was designated RESC-01, for Rat Embryonic Stem cells, C haplotype. A flat-appearing RESC-01 colony is exhibited in **Figure 1B**.

Example 2

This example sets forth some of the identifying characteristics observed for rat embryonic stem cells in culture.

Rat embryonic stem cells obtained in Example 1 were typically rather small. The cells ranged in size from about 10-20 microns, with most cells being about 13 microns. As exhibited in **Figure 1B**, colonies of the cells appeared flat when observed with Nomarski optics. Under optimal culture conditions differentiation of the embryonic stem cells did not occur. Differentiation could be observed initially, as shown in **Figure 1B**, by a surrounding at the periphery of the colony of endoderm cells.

The rat embryonic stem cells had a prominent nucleus containing one or more nucleoli. The cells typically contained a minimal amount of cytoplasm. The cells were demonstrated to be diploid by karyotype analysis. There were no obvious borders between the cells in culture.

The identification of the rat embryonic stem cells was validated by carefully observing the growth of the putative

colony. A rat embryonic stem cell colony exhibited growth and an absence of overt differentiation, as well as an ability to contribute to chimera formation, as described in subsequent examples. These characteristics of the rat embryonic stem cells are indicative of pluripotent stem cells.

Example 3

This example confirms the method of maintaining rat embryonic stem cells in culture and validates the growth of these cells on various substrata.

The RESC-01 cells of Example 1 at passage 6-7 were plated onto 60 mm plastic, gelatin-coated plastic, or plastic dishes precoated with either STO mouse fibroblast feeder layers or HREF fibroblast feeder layers. In this example as well as subsequent examples, feeder cell layers were growth arrested prior to use as described in Example 1.

For these experiments, RESC-01 cells were harvested with trypsin and maintained at 4°C during cell manipulation. About 5 x 10^4 RESC-01 cells were then plated onto feeder layers in DMEM supplemented as previously described for isolation of rat embryonic stem cells. The supplemented DMEM also contained 500 units/ml LIF, to allow growth curves to be obtained. Under these plating conditions, the RESC-01 cells attach within 24 hours as single cells. Duplicate plates were set up for each time point, and the medium was changed daily. The cells were harvested with trypsin and counted with a hemocytometer on days 1, 2, 3, 4, and 5.

As shown by the growth curves in **Figure 2**, the RESC-01 cells grow best on the HREF feeder layers. The RESC-01 cells grew less well on the STO feeder layers, even less well on gelatin-coated plastic, and the worst on plastic. The differences between the growth curves on the different substrata were statistically significant. The cell counts for RESC-01 cells grown on HREF feeder layers were

significantly greater than the cell counts for cells grown on STO feeder layers on day 2 ($p < 0.01$) and day 4 ($p = 0.01$). They were significantly greater than those obtained for cells grown on gelatin-coated plastic on day 2 ($p < 0.001$) and day 5 ($p = 0.03$). Furthermore, they were significantly greater than those obtained for cells grown on plastic on day 2 ($p < 0.001$), day 3 ($p = 0.001$) and day 5 ($p < 0.005$).

Similarly, the cell counts for RESC-01 cells grown on STO feeder layers were significantly greater than the cell counts for cells grown on gelatin-coated plastic on day 5 ($p = 0.04$), and for cells grown on plastic on days 2, 3, 4 and 5 ($p < 0.01$). Also, the cell counts for RESC-01 cells grown on gelatin-coated plastic were significantly greater than the cell counts for cells grown on plastic on day 3 ($p < 0.05$), day 4 ($p = 0.002$), and day 5 ($p < 0.02$).

These experiments corroborate the fact that rat embryonic stem cells can be maintained in culture. Furthermore, the experiments validate that rat embryonic stem cells of the present invention differ from previously described pluripotent embryonic stem cells in exhibiting a preference for growth on the HREF feeder layers on which these cells were derived.

Example 4

This example corroborates the growth and differentiation of rat embryonic stem cells in culture on various substrata.

Duplicate plastic 60 mm plates were prepared for use by coating plates with gelatin, or by plating on growth arrested fibroblast feeder layers. The RESC-01 cells of Example 1 were harvested with trypsin and were maintained at room temperature during cell manipulation. About 5×10^4 RESC-01 cells were plated on HREF feeder layers, STO feeder layers, gelatin-coated plastic, or plastic in medium containing either 500 units LIF/ml or no LIF at all.

Under these plating conditions, the RESC-01 cells attach within 24 hours in clumps of 2 to 3 cells. Cell aggregates were examined with a 20X phase contrast objective. Each day fifty randomly chosen colonies were examined, and the percentage which demonstrated any evidence of differentiation was recorded. Fifty colonies were scored in six determinations made on duplicate plates. A colony in which any differentiated cells (epithelial, mesenchymal, or endodermal morphologies) were observed was scored as differentiated.

In the absence of LIF, when cultured on the feeder layers, the RESC-01 cells demonstrate slow differentiation toward endoderm-like cells. In distinct contrast to the slow differentiation of RESC-01 cells observed on feeder layers, the cells exhibit rapid differentiation when cultured on plastic or gelatin-coated plastic. Specifically, RESC-01 cells grown on plastic or gelatin-coated plastic differentiate into cells which are morphologically distinct from undifferentiated RESC-01 cells. As exhibited in **Figures 1D-F**, under these conditions, the obtained differentiated culture was comprised of round refractile endoderm-like cells on the surface of RESC-01 colonies, flat polygonal epithelial cells, and fusiform-shaped mesodermal cells.

In the presence of LIF, as verified in **Figure 3**, there was a statistically significant difference in the number of RESC-01 colonies which contained differentiated cells on the various substrata. On days 2 and 3 following plating, the least differentiation occurred on HREF feeder layers, followed by STO feeder layers, and gelatin-coated plastic. As expected, plating on plastic led to the most rapid differentiation of colonies, despite the presence of LIF in the medium. However, under these conditions, on day 3 following plating, there was no difference in the proportion of differentiated colonies when the RESC-01 cells were plated on gelatin-coated plastic vs. plastic.

In particular, the proportion of differentiated colonies of RESC-01 cells grown on HREF feeder layers was significantly less than the proportion of differentiated colonies grown on STO feeder layers on day 2 ($p = 0.02$), than the proportion grown on gelatin-coated plastic on days 2, 3, and 4 ($p < 0.001$), and than the proportion grown on plastic on days 2, 3, and 4 ($p < 0.001$). Similarly, the proportion of differentiated colonies of RESC-01 cells grown on STO feeder layers was significantly less than the proportion grown on gelatin-coated plastic on days 2, 3, and 4 ($p < 0.001$), and than the proportion grown on plastic on days 2, 3, and 4 ($p < 0.001$). Moreover, the proportion of differentiated colonies of RESC-01 cells grown on gelatin-coated plastic was significantly less than the proportion grown on plastic on day 2 ($p < 0.003$).

These experiments confirm that the present inventive rat embryonic stem cells can be cultured in vitro and maintained in an undifferentiated, pluripotent state by plating the cells on embryonic fibroblast feeder cell layers or in the presence of LIF.

Example 5

This example corroborates the lack of a negative effect of different concentrations of LIF on growth of rat embryonic stem cells plated on various substrata.

The RESC-01 cells of Example 1 were plated on either HREF feeder layers or gelatin-coated plastic in the presence of a LIF concentration of 0, 100, 500, 1000 or 2000 units/ml.

As shown by the growth curves presented in **Figures 4A-B**, the presence of LIF in the medium at concentrations ranging from 0 to 2000 units/ml did not diminish proliferation of RESC-01 cells on either HREF embryonic fibroblasts or gelatin-coated plastic. Unlike mouse pluripotent embryonic stem cells, there was no growth plateau evidenced for RESC-01 cells at even the highest LIF dose of 2000 units/ml.

These experiments confirm that LIF can be employed in tissue culture medium to maintain rat embryonic stem cells in an undifferentiated, pluripotent state at a concentration up to 2000 units/ml without negatively impacting on proliferation. Since no decrease in proliferation was observed at the even the highest LIF dose, a LIF dose of much greater than 2000 units/ml may be necessary to completely abrogate rat embryonic stem cell proliferation.

Example 6

This example validates the concentration-dependence of LIF-mediated inhibition of differentiation of rat embryonic stem cells plated on various substrata.

The ability to maintain the RESC-01 cells of Example 1 in an undifferentiated state by addition of different concentrations of LIF was investigated using various substrata. The experiments were conducted as in Example 5, except that, instead of counting cells, the percentage of differentiated cells in a random sample was determined.

The addition of various concentrations of LIF to RESC-01 cells growing on either HREF feeder layers or gelatin-coated plastic confirmed the LIF concentration-dependent inhibition of differentiation of RESC-01 cells. As shown in **Figures 5A-B** the differentiation of RESC-01 cells was faster on gelatin-coated plastic than on HREF fibroblasts at all LIF concentrations. No difference was observed between the two substratum conditions at a range of 0 to 100 units LIF/ml. However, significant differences between the two substratum conditions were observed on days 2 and 3 following plating at all tested concentrations greater than 100 units LIF/ml, with the proportion of differentiated colonies decreasing at higher LIF concentrations.

Specifically, the proportion of differentiated colonies of RESC-01 cells grown on HREF in the absence of LIF was significantly greater than the proportion obtained

in the presence of 500 units/ml LIF on days 2 and 3 ($p < 0.001$), than the proportion obtained in the presence of 1000 units/ml LIF on day 2 ($p < 0.001$), and than the proportion obtained in the presence of 2000 units/ml LIF on days 2 and 3 ($p < 0.006$). The proportion of differentiated colonies of RESC-01 cells grown on gelatin-coated plastic in the absence of LIF was significantly greater than the proportion grown in the presence of 500 units/ml of LIF on day 2 ($p < 0.0001$), than the proportion obtained in the presence of 1000 units/ml of LIF on day 2 ($p = 0.007$), and than the proportion grown in the presence of 2000 units/ml of LIF on day 2 ($p = 0.0001$).

These results corroborate that the present inventive rat embryonic stem cells can be maintained in the undifferentiated state using LIF. Moreover, these results verify the concentration- and substratum-dependent ability of LIF to inhibit the differentiation of the rat embryonic stem cells. The proportion of differentiated cells decreases as the concentration of LIF increases. Also, LIF more effectively retards differentiation of the rat embryonic stem cells when these cells are plated on HREF feeder layers, as opposed to on gelatin-coated plastic. This synergistic effect of LIF employed with HREF feeder layers has not been reported for embryonic stem cells of other species.

Example 7

This example confirms the ability of rat embryonic stem cells to spontaneously differentiate in suspension culture, resulting in the formation of embryoid bodies, or cystic structures comprised of several cell layers, which are reminiscent of the early embryo.

For these experiments, the RESC-01 cells of Example 1 were lightly trypsinized, and clumps of cells were transferred with a wide-bore pipette into 100 mm bacteriological Petri dishes (Baxter, Deerfield, IL) for 30 minutes to allow attachment of fibroblasts. The unattached

RESC-01 cells were then placed in sterile 100 mm plastic bacteriological Petri dishes containing 10 ml of DMEM, which was supplemented as described in Example 1. The cellular aggregates were cultured in suspension for 4 to 5 days without further addition of any medium. The cultures were passaged after this amount of time by first settling the simple embryoid bodies in a conical tube. The exhausted medium was then aspirated off, the embryoid bodies were split into three plates, and 10 to 12 ml of fresh medium were added to each plate. The embryoid bodies were incubated for another 4 to 5 days, with the cultures being refed every other day. On the days when the medium was not replaced, 5 ml of fresh medium was added.

Under these conditions, the RESC-01 cells spontaneously formed cystic bodies in culture, which were capable of further growth in suspension culture. As demonstrated in **Figures 1G-H**, the cystic bodies differentiated within 4 to 6 days into structures identical to simple embryoid bodies. After 7 days, some of the rat cystic embryoid bodies acquired complex shapes with cystic fluid-filled cavities comprised of two cell layers (**Figures 1G-H**): one endoderm-like and the other ectoderm-like. Several of these rat embryoid bodies were observed by phase contrast microscopy to begin rhythmic contractions similar to those produced with mouse embryoid bodies (Sanchez et al., J. Biol. Chem., 266, 22419-22426 (1991)). These structures can be cultured for many weeks.

These experiments confirm that cystic embryoid bodies are easily established from the present inventive rat embryonic stem cells in culture. The experiments additionally confirm that the embryoid bodies, like mouse embryoid bodies, can undergo further developmental changes in culture. The results of these experiments, coupled with the demonstration in previous examples of the ability of rat embryonic stem cells to differentiate to various cell types, evidence the pluripotency of the present inventive rat embryonic stem cells.

Example 8

This example demonstrates that rat embryonic stem cells can participate in chimera formation following microinjection into blastocyst stage embryos.

The selection of the strain used for chimera formation is an important consideration because certain inbred strains may yield fewer embryos, and the methodology requires an appropriately marked strain such that chimeric and non-chimeric rats can be distinguished. Moreover, certain pairings of strains could conceivably result in the generation of sterile offspring. Accordingly, for these experiments, the Holtzman strain was selected as host for chimera formation.

The RESC-01 cells of Example 1 were microinjected into Holtzman strain rat blastocysts isolated as in Example 1. For these experiments, the RESC-01 cells were trypsinized for 3 minutes at 37°C, and were subsequently pipetted gently for 2 minutes through a narrow bore pipette to insure a single cell suspension. Following inactivation of the trypsin through addition of 2-3 ml of DMEM, 6 ml of flushing medium (Spindle et al., J. Exp. Zoology, 186, 305-318 (1973)) or M2 (Specialty Media, Lavallett, NJ) were added. The cells were pelleted by centrifugation. Approximately 1000 to 5000 cells were resuspended in about 25 μl of flushing medium or M2 in a plastic 60 mm Petri plate fitted into a cooling stage at 10°C, and covered with sterile mineral oil.

The RESC-01 cells were injected into rat blastocoel cavities as described for injection into mouse blastocoel cavities (Bradley, In: Teratocarcinomas and embryonic stem cells: a practical approach, E.J. Robertson, ed. (Oxford: IRL Press, 1987) 113-151), with necessary modifications being made to accommodate species-specific differences in embryological processes. Namely, rat blastocysts appropriate for injection were obtained on day 4.5 of pregnancy as compared with day 3.5 for mice. The rat morula stage embryo differs distinctly from that of the

mouse in being planar in shape (Yamamura et al., Dev. Genet., 2, 131-146 (1981); Weinberg et al., J. Cell Sci., 89, 423-431 (1988)). This geometry was maintained in the rat blastocyst, which as demonstrated in Figures 6A-C, was characteristically ovoid in shape. As a result of the characteristic ovoid shape of blastocysts isolated from the rat, the blastocyst was expanded prior to microinjection of RESC-01 cells for rat chimera formation.

The expansion of rat blastocysts was accomplished by incubating the blastocyst at 37°C in 5% CO_2 atmosphere for about two hours in Brinster's medium (Brinster, In: Pathways to Conception (Springfield: Charles G. Thomas Publishing Company, 1971)) that had been modified to facilitate expansion of the rat blastocyst, and in particular, the Holtzman strain blastocyst. The modified medium was prepared by adding with stirring to 75 ml of nanopure water in a 100 ml volumetric flask the following tissue culture grade reagents: 554.5 mg NaCl, 35.6 mg KCl, 16.2 mg KH_2PO_4, 14.3 mg anhydrous $MgSO_4$, 210.5 mg $NaHCO_3$, 100 mg dextrose, 5 mg streptomycin sulfate, 6 mg penicillin G, 100 mg albumin (BSA Pentax fraction V), 5.6 mg sodium pyruvate, and 0.38 ml of D,L-sodium lactate. Following the addition of 18.8 mg of $CaCl_2$, the medium was brought to a final volume of 100 ml.

For microinjection, the expanded blastocysts were placed in the microdrop of medium along with the RESC-01 cells. From 10 to 30 RESC-01 cells were injected into the blastocoel using a DeFunbrune pump. After the blastocysts were injected, they were transferred to the uteri of Holtzman strain surrogate mothers on day 3.5 of pseudopregnancy. Pseudopregnancy in the surrogate rats was established by mechanical stimulation of the cervix during estrus (Ng et al., In: Current Topics in Developmental Biology, R.A. Pederson, ed. (San Diego: Academic Press, 1992) 235-274). Pseudopregnancy was alternatively established as for mice, by mating female rats with sterile males and selecting females exhibiting vaginal copulation

plugs (plug discovery = day 0.5). The timing of pseudopregnancy was established by noting the day of onset of leukocytes in the vaginal smear (Ng et al., supra). The surrogate mothers were allowed to deliver naturally.

The procedures developed for rat and outlined herein resulted in a pregnancy rate of 79% and a live birth rate of 39%. Over the course of several experiments, eighty-nine pups were obtained. Eighty-three of the pups were albino, or of the non-chimeric, Holtzman strain. As exhibited in **Figure 7**, six of the pups (two males and four females) were born with a patchy mixture of albino, black and agouti coat colors. These six pups were found to vary from approximately 10% to 80% pigmented coat. All six of the pups displayed posterior pigmentation, which is not observed for hooded PVG or albino Holtzman rats. The six pups contained patches of agouti and black in the pigmented areas. Two of the pups had patches of pigmentation on the head, whereas the PVG coat pattern always consists of pigmentation over the entire head. One of the pups had bilateral pigmentation on the dorsal surface of the distal forelimbs. These patterns observed for the six pups differ distinctively from the patterns observed for either the Holtzman or PVG strain of rats. Specifically, the Holtzman strain is characterized by an albino coat pattern. The PVG strain of black hooded rats is characterized by a complete black coat color in the head and dorsal black pigmentation in the trunk. Accordingly, the coat color patterns observed for the six pups could only have occurred as a result of chimera formation between the injected RESC-01 cells derived from the PVG strain, and the Holtzman strain blastocyst. Thus the patterns represent the effects of mixing in the epidermis and dermis of both Holtzman and PVG cells.

Moreover, the patterns observed in the rat RESC-01 cell chimeras are consistent with those previously reported in aggregation chimeras formed by amalgamation of eight-cell embryos from strains genetically similar to the

strains employed (Yamamura et al., Dev. Genet., 2, 131-146 (1981)). Namely, aggregation chimeras produced between completely black and black hooded strains, or between completely black and albino hooded strains, showed large spots and did not demonstrate stripes or stippled patterns. Further, aggregation chimeras produced between completely black and albino hooded strains showed patchy mosaicism in the head similar to the RESC-01 cell chimeras. These similarities provide convincing evidence that the RESC-01 cells contributed to chimera formation.

In terms of verification of the method of blastocyst expansion, the population of 89 pups obtained included 46 pups (8 litters) obtained from injections of blastocysts which were not fully expanded. There were no chimeras among these 46 pups. Thirty pups (4 litters) were derived from injections of blastocysts which were fully expanded. Five of these thirty pups were chimeric, and at least one chimera was obtained in each of the four litters. The remaining chimera was from a group of 13 pups (3 litters), which were derived from injections of blastocysts, only some of which were fully expanded. Thus, in the best series of experiments, 17% (or 5/30) of live births were chimeric.

In terms of verification of germ line chimerism, appropriate breeding experiments can be performed. For example, obtained chimera can be mated with the blastocyst donor strain, in this case a Holtzman strain rat, and if the embryonic stem cells have colonized the germ line, then at least some of the offspring should evidence the hooded PVG coat pattern of the pluripotent embryonic stem cells. In situations where the obtained chimeras are sterile, which may be the result of the present pairing between the PVG strain and Holtzman strain, germ line chimeras can be obtained using a different strain as blastocyst donor, or by isolating pluripotent embryonic stem cells from a different strain.

These results, therefore, validate the effectiveness of the present inventive approach for expanding blastocysts prior to introduction of rat embryonic stem cells and chimera formation, and in particular, the Holtzman strain blastocysts. Namely, Holtzman strain rat blastocysts expanded using this approach appear more likely to yield chimeras. The experiments further verify the pluripotency of the present inventive rat embryonic stem cells, as well as the method of chimera generation in rats, by corroborating the ability of the cells to form chimeras with normal rats.

Example 9

This example describes an alternative method of introducing rat pluripotent embryonic stem cells into multicellular rat embryos by coculturing rat embryos with the stem cells, as well as the use of this method in the generation of chimeric rats.

The RESC-01 cells of Example 1 were prepared as for microinjection of blastocysts, in supplemented DMEM medium containing LIF at a concentration of 2000 units/ml, as described in Example 8. The cell suspension was placed in a plastic culture dish for 10 to 20 minutes to allow fibroblasts to attach. Unattached cells were collected by centrifugation and resuspended in DMEM containing 5% FBS and 23 mM sodium lactate (Sigma L-4263). Aliquots of 15 μl were placed in drops onto a 60 mm tissue culture plate, and 10 μl of the cell suspension was added to each drop to obtain about 8.5×10^3 RESC-01 cells per drop. The drops were overlaid with light mineral oil and equilibrated at 37°C and 6% CO_2.

Morula stage rat embryos were isolated from pregnant rats at day 3.5 of pregnancy (sperm discovery = day 0.5) by flushing the oviduct with T6'. The zona pellucida was removed from the embryos by brief (30 to 60 seconds) incubation in modified T6' solution containing 1 g/l sodium bicarbonate and 10 g/l polyvinyl pyrrolidine, and from

which penicillin/streptomycin, phenol red, BSA and HEPES had been omitted. The final pH of the modified T6' solution was adjusted to 2.5, and the osmolality was adjusted with water to 310 mOsm/kg H_2O. Following this treatment, the embryos were placed in the drops of the RESC-01 cells for about 20 minutes to 2 hours until 3-10 RESC-01 cells attached to the embryos. The embryos were subsequently transferred to an organ culture dish which had been coated with heat-inactivated rat serum, and were rinsed with Markert's modification of Whittingham's medium that had been adjusted with water to an osmolality of 295 mOsm/kg H_2O. The embryos were cultured in the medium for 20 to 26 hours, and then transferred to pseudopregnant surrogate mothers, as in Example 8.

As for the method of microinjecting rat pluripotent embryonic stem cells into rat blastocysts, it is expected that rat chimeras will similarly be obtained by the outlined method of coculturing multicellular rat embryos with rat pluripotent embryonic stem cells prior to transfer to pseudopregnant rats. It is further anticipated that this latter approach may prove more effective than microinjection for generation of chimeric rats when certain strains of rat are utilized.

Example 10

This example describes a method of producing transgenic chimeras using the rat pluripotent embryonic stem cells.

In Examples 8 and 9, methods of producing a rat chimera using rat pluripotent embryonic stem cells were described. In the methods described, the stem cells were not modified in any fashion prior to incorporation in the rat blastocyst or earlier stage embryo. However, the rat pluripotent embryonic stem cells can be modified prior to incorporation into the embryo or blastocyst, for example, by incorporation into these cells of exogenous DNA.

For this purpose, a commercially available expression vector containing β-galactosidase coding sequences under the control of a RSV promoter (Stratagene, La Jolla, CA) can be employed. The lacZ sequences in this vector are separated from the promoter by a linker sequence containing appropriate restriction sites for subcloning of genes or gene fragments. The expression vector is transfected into the RESC-01 cell line. Transfectants are confirmed, and expression of β-galactosidase verified, by replica plating colonies and staining for β-galactosidase activity (S. Gal, Methods Enzymol., 151, 104 (1987); Sanes et al., Embo J., 5, 3133 (1986)). Colonies which express β-galactosidase are then selected and expanded. The resultant cell line is introduced into a rat blastocyst or early stage embryo, and subsequently transferred to a pseudopregnant rat, as set forth in Examples 8 and 9.

The transgenic chimeras obtained using this approach can be verified by simple observation of phenotypic properties, as described in Example 8, as well as by fixing tissue sections in glutaraldehyde solution, and analyzing sections for β-galactosidase activity (Sanes et al., Embo J., 5, 3133 (1986)). Additional experiments can also be performed, such as Southern and Northern hybridization, or Western blotting, to verify the presence or expression of the exogenous DNA sequences in the chimeric host.

Using the method outlined, it is expected that rat pluripotent embryonic stem cells which have been altered in some fashion will be obtained. These stem cells can further be employed in the method of Examples 8 or 9 to generate transgenic rats.

Example 11

This example describes a method of obtaining rats which are homozygous for DNA sequences introduced into or altered in the rat pluripotent embryonic stem cells.

The chimeras obtained by the methods outlined in Examples 8-10 are mosaics, comprised of cells inherited

from the RESC-01 cells of Example 1, as well as cells inherited from the host blastocyst or embryo. The rats produced in Example 10 contain the introduced or altered DNA in the heterozygous state. Rats containing the introduced or altered DNA of the rat pluripotent embryonic stem cells in the homozygous state can be produced by mating with each other germ line chimeras obtained by the methods of Examples 8, 9 or 10, as described in Example 8. Under these conditions, one fourth of the offspring will typically be homozygous for the introduced or altered DNA.

As long as the gene alteration is not a recessive lethal mutation, the new strain of rat can be bred to carry the alteration in the homozygous state. If the loss or gain of function of the gene causes perinatal mortality, the altered gene can be maintained in the heterozygous state.

It is expected, therefore, that transgenic rats which are homozygous for a particular allele can be obtained using the outlined method.

All documents, including publications, patents, and patent applications, cited herein are hereby incorporated by reference to the same extent as if each individual document were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

While this invention has been described with emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that the preferred embodiments may be varied. It is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the appended claims.

WHAT IS CLAIMED IS:

1. Substantially pure rat pluripotent embryonic stem cells.

2. The embryonic stem cells of claim 1, wherein said cells are isolated from in vitro treatment of rat preimplantation embryos.

3. The embryonic stem cells of claim 2, wherein said cells:

(a) are capable of growth in in vitro culture,

(b) are comprised of a prominent nucleus containing one or more nucleoli,

(c) range in size from about 10-20 microns, and

(d) are capable of contributing to rat chimera formation.

4. The embryonic stem cells of claim 1, which comprise genetic material with at least one change therein.

5. The embryonic stem cells of claim 4, wherein said change is selected from the group consisting of addition of a DNA segment, rearrangement of a DNA segment, deletion of a DNA segment, replacement of a DNA segment, methylation of unmethylated DNA, demethylation of methylated DNA, and introduction of a DNA lesion.

6. The embryonic stem cells of claim 5, wherein said change is selected from the group consisting of addition of a DNA segment and rearrangement of a DNA segment.

7. A preimplantation embryo into which has been introduced in vitro one or more stem cells according to claim 1.

8. An embryonic cell into which has been introduced in vitro a nucleus of a stem cell according to claim 1.

9. A preimplantation embryo into which has been introduced in vitro one or more stem cells according to claim 4.

10. An embryonic cell into which has been introduced in vitro a nucleus of a stem cell according to claim 4.

11. A chimeric rat which is the progeny of a preimplantation embryo according to claim 7.

12. A chimeric rat which is the progeny of an embryonic cell according to claim 8.

13. A transgenic rat which is the progeny of a preimplantation embryo according to claim 9.

14. A transgenic rat which is the progeny of an embryonic cell according to claim 10.

15. A method of obtaining pluripotent embryonic stem cells according to claim 1, which comprises:

(a) isolating a rat preimplantation embryo at a stage of development from which embryonic stem cells can be obtained,

(b) culturing said preimplantation embryo on a feeder cell layer,

(c) maintaining said culture under conditions sufficient to allow said preimplantation embryo to attain a size and stage of development appropriate for isolation of embryonic stem cells,

(d) disrupting said preimplantation embryo,

(e) culturing the fragments obtained from said disruption on a new feeder cell layer, and

(f) maintaining said culture or disrupting said fragments and culturing on a new feeder cell layer until substantially pure pluripotent embryonic stem cells are obtained.

16. The method of claim 15, wherein said feeder cell layer is comprised of rat embryonic fibroblasts.

17. The method of claim 15, wherein said preimplantation embryo is a blastocyst.

18. The method of claim 17, wherein said blastocyst is from a PVG strain of rat.

19. A method of culturing the pluripotent embryonic stem cells of claim 1, which comprises maintaining cells in the presence of leukemia inhibitory factor or culturing cells on a feeder cell layer.

20. A method of incorporating into a rat preimplantation embryo one or more pluripotent embryonic stem cells of claim 1, which comprises contacting said preimplantation embryo with said pluripotent embryonic stem cells under conditions such that said incorporation is effected.

21. The method of claim 20, wherein said contacting is accomplished by:

(a) preparing said pluripotent embryonic stem cells in a suspension suitable for injection, and

(b) injecting the suspension into said preimplantation embryo.

22. The method of claim 21, wherein said preimplantation embryo is expanded prior to injecting said suspension.

23. The method of claim 22, wherein said preimplantation embryo is expanded by incubation in a cell culture medium comprised of a carbon source, minerals, buffers, proteins, carboxylic acids, and carboxylic acid derivatives.

24. The method of claim 23, wherein said incubation is carried out for at least about one hour.

25. The method of claim 24, wherein said preimplantation embryo is a blastocyst.

26. The method of claim 20, wherein said preimplantation embryo is from a Holtzman strain of rat.

27. The method of incorporating into a rat preimplantation embryo one or more pluripotent embryonic stem cells of claim 1, which comprises coculturing said rat preimplantation embryo with said pluripotent embryonic stem cells under conditions such that said incorporation is effected.

28. A method of introducing into a rat embryonic cell a nucleus of a pluripotent embryonic stem cell of claim 1, which comprises replacing the pronuclei of a fertilized rat egg with the nucleus from said pluripotent embryonic stem cell.

29. A method of producing a chimeric rat which comprises transferring to the uterus of a pseudopregnant rat the preimplantation embryo of claim 7.

30. The method of claim 29, wherein said pseudopregnant rat is of the Holtzman strain.

31. A method of producing a chimeric rat which comprises transferring to the uterus of a pseudopregnant rat an egg prepared from the method of claim 28.

32. The method of claim 20, wherein said stem cells comprise genetic material with at least one change therein.

33. The method of claim 32, wherein said change is selected from the group consisting of addition of a DNA segment, rearrangement of a DNA segment, deletion of a DNA segment, replacement of a DNA segment, methylation of unmethylated DNA, demethylation of methylated DNA, and introduction of a DNA lesion.

34. The method of claim 33, wherein said change is selected from the group consisting of addition of a DNA segment and rearrangement of a DNA segment.

35. The method of claim 32, wherein said contacting is accomplished by:

(a) preparing said pluripotent embryonic stem cells in a suspension suitable for injection, and

(b) injecting the suspension into said preimplantation embryo.

36. The method of claim 35, wherein said preimplantation embryo is expanded prior to injecting said suspension.

37. The method of claim 36, wherein said preimplantation embryo is expanded by incubation in a cell culture medium comprised of a carbon source, minerals, buffers, proteins, carboxylic acids, and carboxylic acid derivatives.

38. The method of claim 37, wherein said incubation is carried out for at least about one hour.

39. The method of claim 38, wherein said preimplantation embryo is a blastocyst.

40. The method of claim 27, wherein said stem cells comprise genetic material with at least one change therein.

41. The method of claim 40, wherein said change is selected from the group consisting of addition of a DNA segment, rearrangement of a DNA segment, deletion of a DNA segment, replacement of a DNA segment, methylation of unmethylated DNA, demethylation of methylated DNA, and introduction of a DNA lesion.

42. The method of claim 41, wherein said change is selected from the group consisting of addition of a DNA segment and rearrangement of a DNA segment.

43. The method of claim 28, wherein said stem cells comprise genetic material with at least one change therein.

44. The method of claim 43, wherein said change is selected from the group consisting of addition of a DNA segment, rearrangement of a DNA segment, deletion of a DNA segment, replacement of a DNA segment, methylation of unmethylated DNA, demethylation of methylated DNA, and introduction of a DNA lesion.

45. The method of claim 44, wherein said change is selected from the group consisting of addition of a DNA segment and rearrangement of a DNA segment.

46. A method of producing a chimeric rat which comprises transferring to the uterus of a pseudopregnant rat the preimplantation embryo of claim 9.

47. A method of producing a chimeric rat which comprises transferring to the uterus of a pseudopregnant rat the embryonic cell of claim 10.

48. A medium for in vitro culture of mammalian cells, which comprises a cell culture medium supplemented with serum, antibiotics, amino acids, nucleosides, and β-mercaptoethanol.

49. The medium of claim 48 which further comprises leukemia inhibitory factor.

50. The chimeric rat of claim 11, wherein said rat is a germ line chimera.

51. The chimeric rat of claim 11, wherein said rat is a somatic cell chimera.

52. The chimeric rat of claim 12, wherein said rat is a germ line chimera.

53. The chimeric rat of claim 12, wherein said rat is a somatic cell chimera.

54. The transgenic rat of claim 13, wherein said rat is a germ line chimera.

55. The transgenic rat of claim 13, wherein said rat is a somatic cell chimera.

56. The transgenic rat of claim 14, wherein said rat is a germ line chimera.

57. The transgenic rat of claim 14, wherein said rat is a somatic cell chimera.

58. The method of claim 46, wherein said chimeric rat is a germ line chimera.

59. The method of claim 46, wherein said chimeric rat is a somatic cell chimera.

60. The method of claim 47, wherein said chimeric rat is a germ line chimera.

61. The method of claim 47, wherein said chimeric rat is a somatic cell chimera.

62. A method of producing a rat containing a particular allele in the homozygous state, which comprises mating with each other the chimeric rats prepared from the method of claim 58.

63. A method of producing a rat containing a particular allele in the homozygous state, which comprises mating with each other the chimeric rats prepared from the method of claim 60.

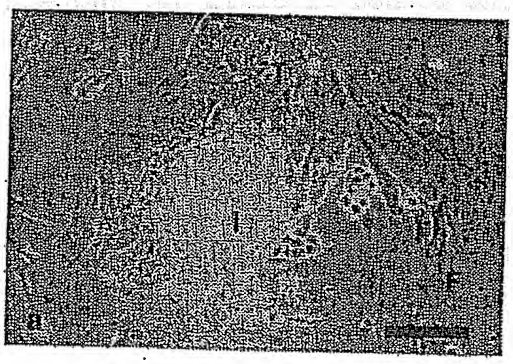


FIG. 1A

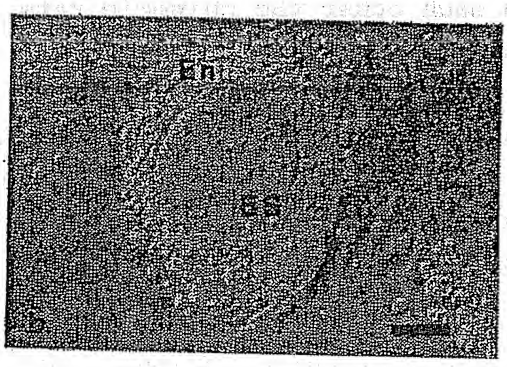


FIG. 1B

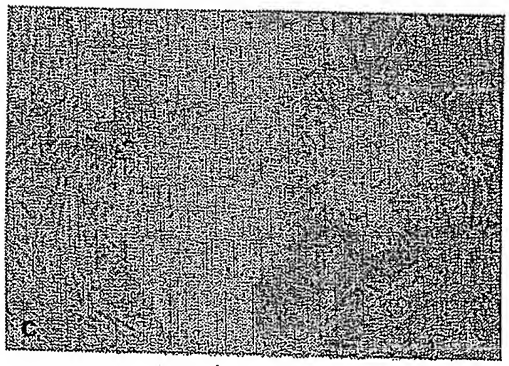


FIG. 1C

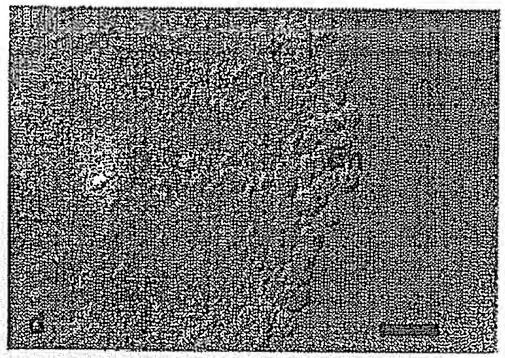


FIG. 1D

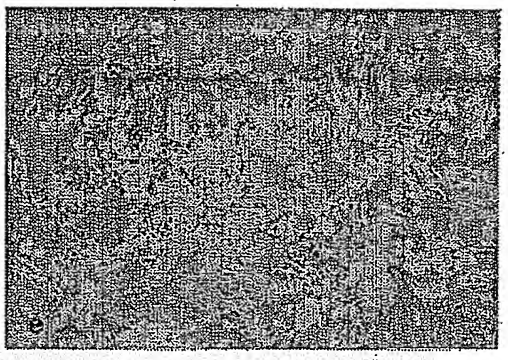


FIG. 1E

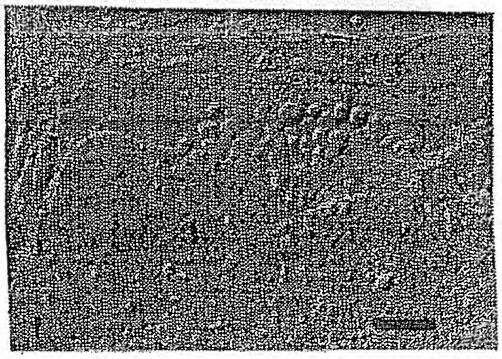


FIG. 1F

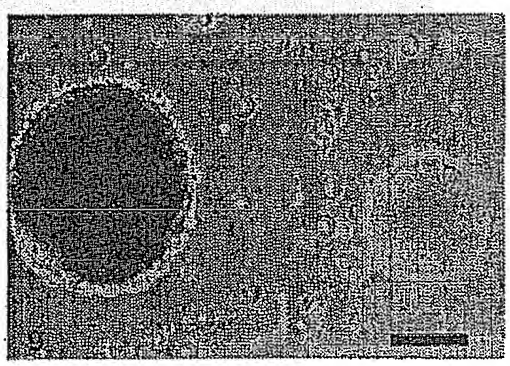


FIG. 1G

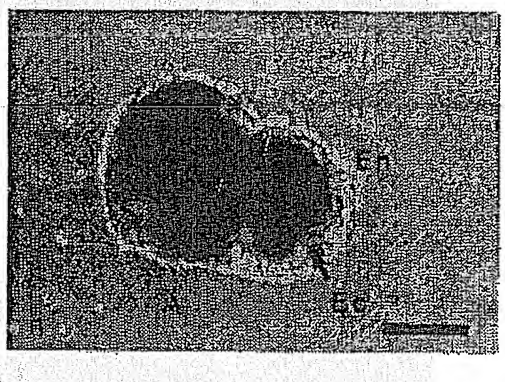


FIG. 1H

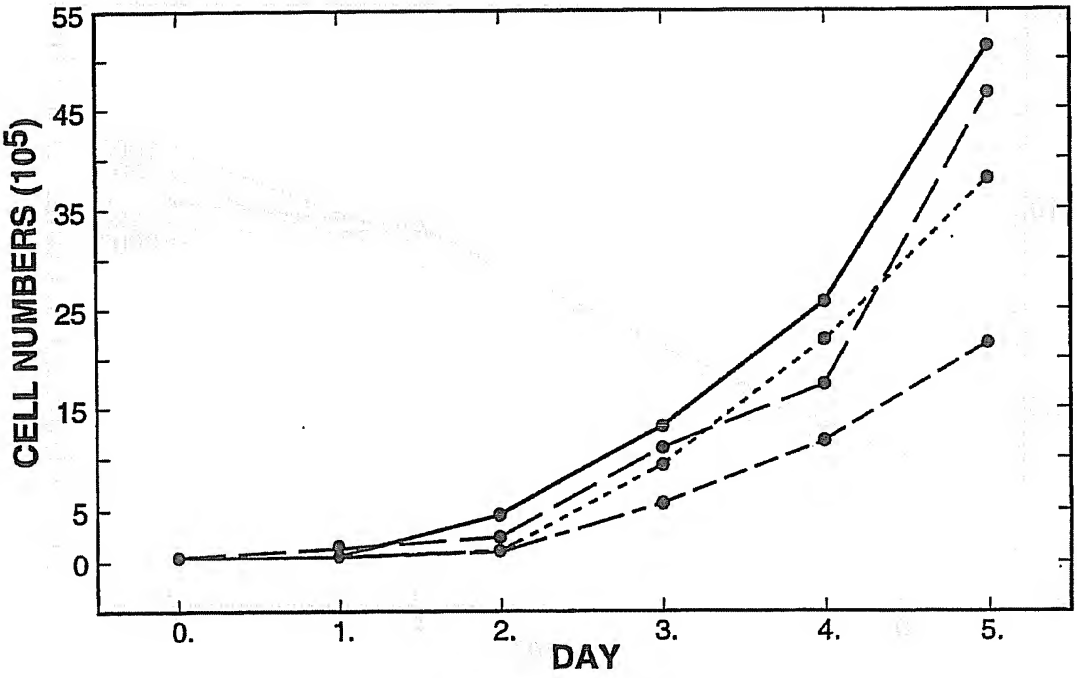

CELL NUMBERS (10^5)
55
45
35
25
15
5
0
0.
1.
2.
3.
4.
5.
DAY

FIG. 2

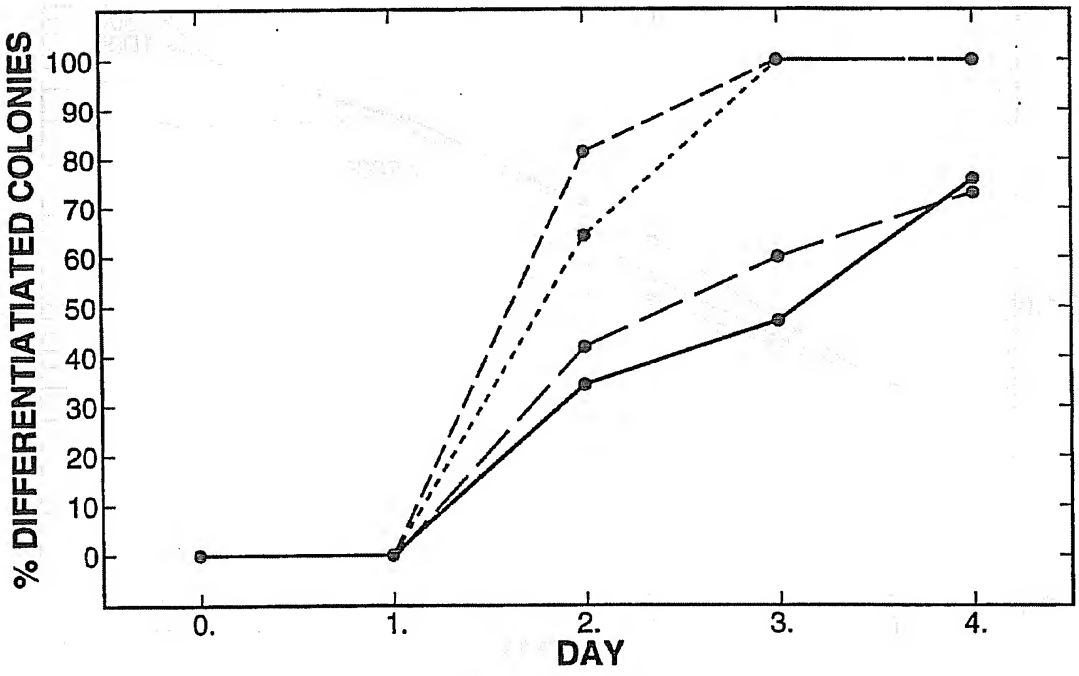
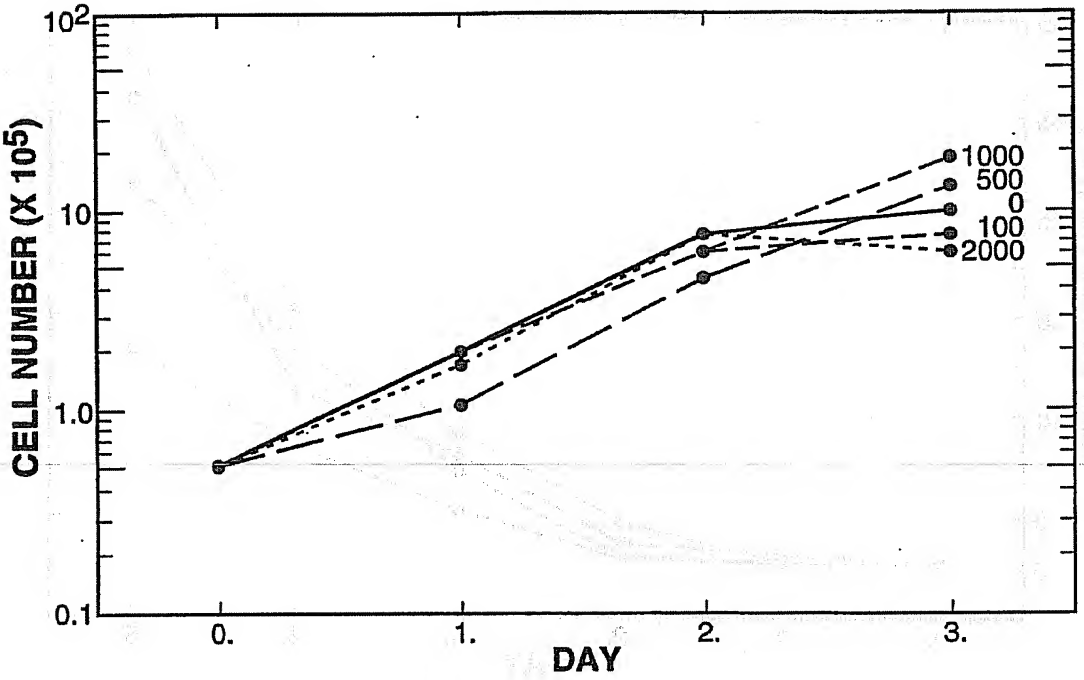

% DIFFERENTIATED COLONIES
100
90
80
70
60
50
40
30
20
10
0
0.
1.
2.
3.
4.
DAY

FIG. 3


10^2
10
1.0
0.1
CELL NUMBER (X 10^5)
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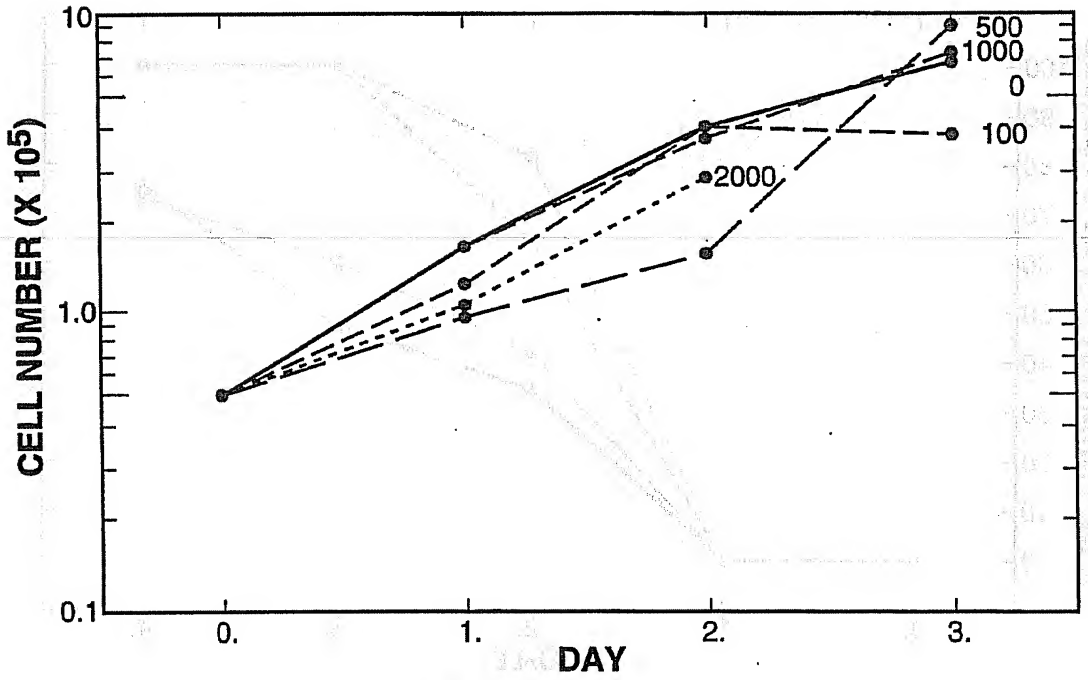
1.
2.
3.
DAY
1000
500
0
100
2000

FIG. 4A


10
1.0
0.1
CELL NUMBER (X 10^5)
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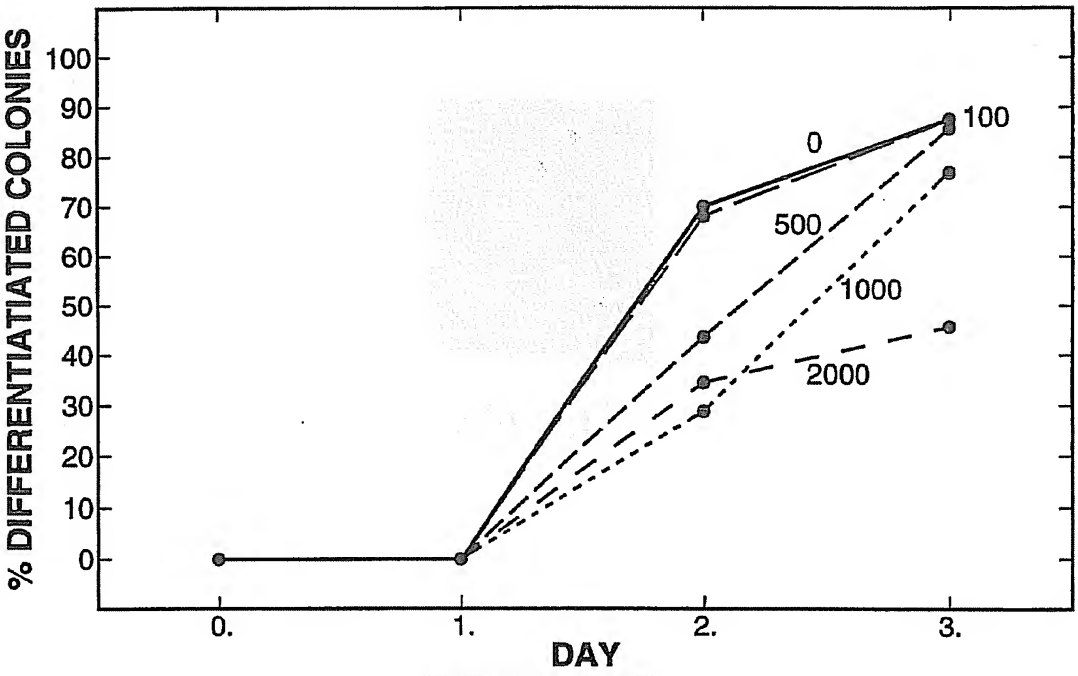
1.
2.
3.
DAY
500
1000
0
100
2000

FIG. 4B


% DIFFERENTIATED COLONIES
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90
80
70
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30
20
10
0
0.
1.
2.
3.
DAY
0
100
500
1000
2000

FIG. 5A

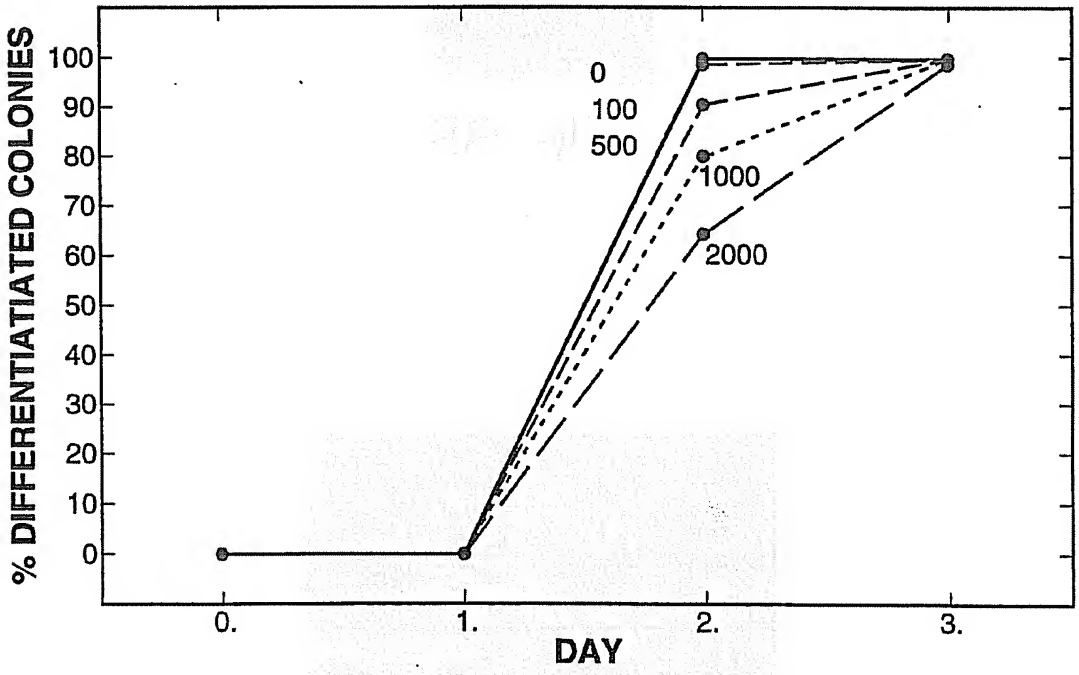
% DIFFERENTIATED COLONIES
100
90
80
70
60
50
40
30
20
10
0
0.
1.
2.
3.
DAY
0
100
500
1000
2000

FIG. 5B

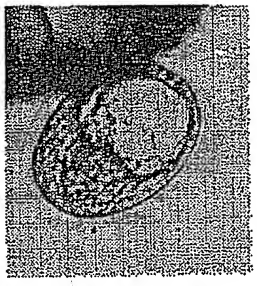

FIG. 6A

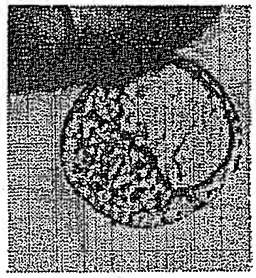

FIG. 6B

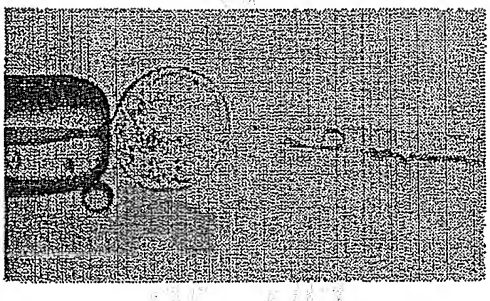

FIG. 6C

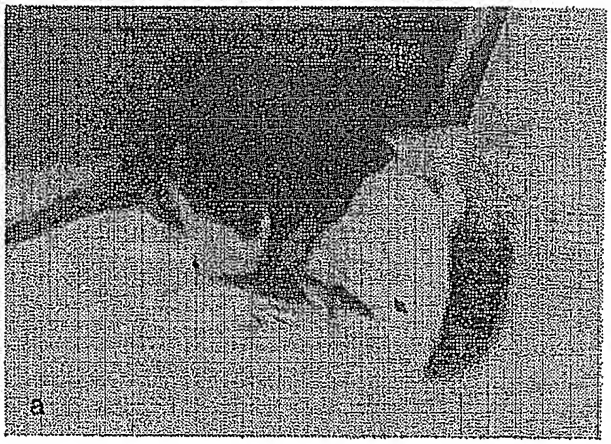

FIG. 7A

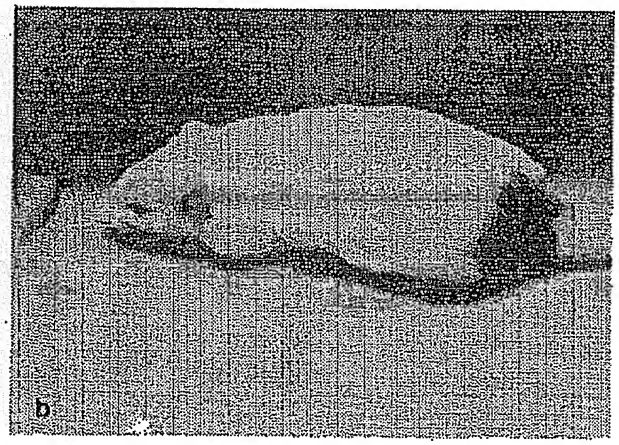

FIG. 7B

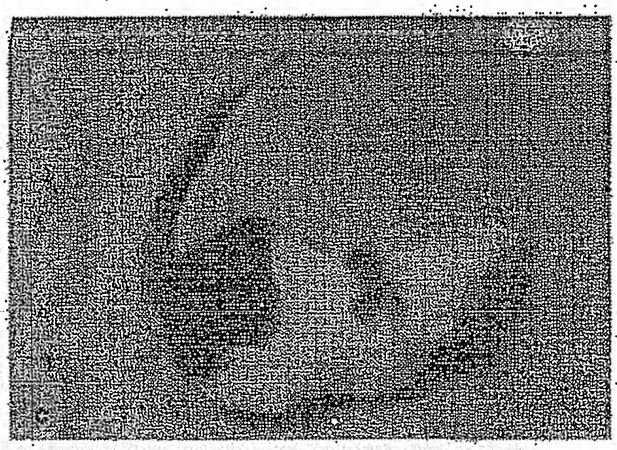

FIG. 7C

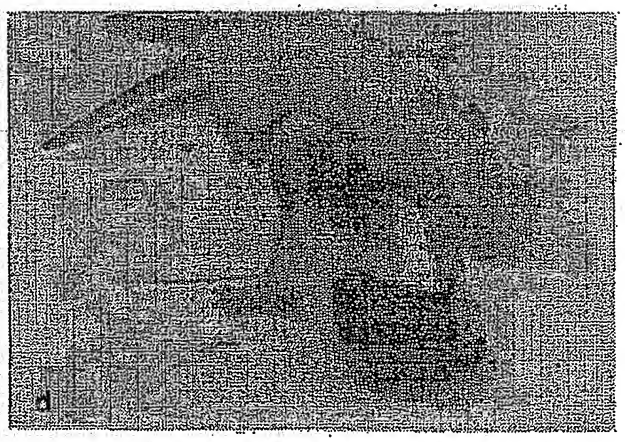

FIG. 7D

INTERNATIONAL SEARCH REPORT

Interna tl Application No
PCT/US 94/09787

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 A01K67/027 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 375 406 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 27 June 1990 see page 15, line 36 - page 20, line 22; claims 36-40 ---	1-47, 50-63
X	WO,A,91 19796 (BAYLOR COLLEGE OF MEDICINE) 26 December 1991 see page 15, line 10 - page 20, line 15 see page 41, line 19 - page 43, line 15 see page 45, line 21 - page 58, line 33 ---	1-47, 50-63
X	WO,A,92 03917 (GENPHARM INTERNATIONAL) 19 March 1992 see page 7, line 30 - page 9, line 7 see page 10, line 5 - page 23, line 10 --- -/--	1-47, 50-63

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 December 1994	28.12.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 223 610 (BURTON ET AL) 29 June 1993 see column 12, line 38 - column 16, line 63	1-47, 50-63
X	WO,A,90 01541 (AMRAD CORPORATION LIMITED) 22 February 1990	48,49
A	see page 6, line 4 - page 7, line 20	1-47, 50-63
X	THERIOGENOLOGY, vol.33, no.4, April 1990 pages 901 - 913 STROJEK ET AL 'A METHOD FOR CULTIVATING MORPHOLOGICALLY UNDIFFERENTIATED EMBRYONIC STEM CELLS FROM PORCINE BLASTOCYSTS'	48
A	see page 901,abstract see page 903, paragraph 3	1-47, 49-63
X	WO,A,90 03432 (ANIMAL BIOTECHNOLOGY CAMBRIDGE LIMITED ET AL) 5 April 1990 cited in the application	48
A	see page 8, paragraph 2 - page 13, paragraph 4	1-47, 49-63
X	WO,A,90 02183 (GENETICS INSTITUTE,INC. ET AL) 8 March 1990 see page 15, line 20 - page 16, line 14	48,49
X	EP,A,0 341 065 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 8 November 1989 see page 38, line 15 - page 39, line 13	48
A	WO,A,93 11228 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 10 June 1993 see page 11, paragraph 2 - page 13, paragraph 1	
A	WO,A,93 01272 (UNIVERSITY OF LEICESTER) 21 January 1993 see page 1, paragraph 1 - page 3, paragraph 1	
P,X	DEVELOPMENTAL BIOLOGY, vol.163, no.1, May 1994 pages 288 - 292 IANNACOONE ET AL 'PLURIPOTENT EMBRYONIC STEM CELLS FROM THE RAT ARE CAPABLE OF PRODUCING CHIMERAS' see the whole document	1-63

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE FASEB JOURNAL, vol.8, no.4, 15 March 1994 page A535 BRENIN ET AL 'RAT EMBRYONIC STEM CELLS:CHARACTERIZATION,CHIMERA PRODUCTION,AND EVALUATION OF PLURIPOTANCY' see abstract no.3098	1-47, 50-63
O,P,X	PROCEEDNGS OF THE EIGHTY-FIFTH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol.35, March 1994, SAN FRANCISCO,CA,USA page 612 BRENIN ET AL 'THE DERIVATION AND CHARACTERIZATION OF RAT EMBRYONIC STEM CELLS' see abstract no.3646	1-47, 50-63
O,P,X	ABSTRACTS.THE AMERICAN SOCIETY FOR CELL BIOLOGY THIRTY-THIRD ANNUAL MEETING, December 1993, NEW ORLEANS,LO,USA & MOLECULAR BIOLOGY OF THE CELL, vol.4, October 1993 page 252A IANNACCONE ET AL 'DERIVATION OF PLURIPOTENT EMBRYONIC STEM CELLS FROM THE RAT' see abstract no.1463	1-47, 50-63

INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/US 94/09787

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

See Annex

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Intern al Application No
PCT/US 94/09787

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0375406	27-06-90	AU-B-	627256	20-08-92
		AU-A-	4701589	28-06-90
		CA-A-	2006011	21-06-90
		CN-A-	1046936	14-11-90
		JP-A-	3094626	19-04-91
		WO-A-	9006992	28-06-90
WO-A-9119796	26-12-91	AU-B-	654284	03-11-94
		AU-A-	8182391	07-01-92
		EP-A-	0535144	07-04-93
WO-A-9203917	19-03-92	AU-A-	8649791	30-03-92
		CA-A-	2090473	01-03-92
		EP-A-	0546091	16-06-93
		JP-T-	6506105	14-07-94
US-A-5223610	29-06-93	NONE		
WO-A-9001541	22-02-90	AU-B-	623922	28-05-92
		AU-A-	4059089	05-03-90
		EP-A-	0380646	08-08-90
		JP-T-	3503241	25-07-91
		US-A-	5166065	24-11-92
WO-A-9003432	05-04-90	AU-B-	650616	30-06-94
		AU-A-	4321789	18-04-90
		EP-A-	0435928	10-07-91
		JP-T-	4500909	20-02-92
WO-A-9002183	08-03-90	NONE		
EP-A-0341065	08-11-89	US-A-	5011828	30-04-91
		AT-T-	109155	15-08-94
		AU-B-	620398	20-02-92
		AU-A-	3399889	09-11-89
		AU-B-	634679	25-02-93
		AU-A-	8834591	13-02-92
		CA-A-	1318666	01-06-93
		DE-D-	68917012	01-09-94
		JP-A-	2085293	26-03-90

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Intern al Application No
PCT/US 94/09787

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0341065		US-A-	5136030	04-08-92
WO-A-9311228	10-06-93	AU-A-	3233293	28-06-93
		CA-A-	2125161	10-06-93
		EP-A-	0619838	19-10-94
WO-A-9301272	21-01-93	AU-A-	2230992	11-02-93
		CA-A-	2112897	21-01-93
		EP-A-	0594660	04-05-94
		JP-T-	6509469	27-10-94